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ACTA CYTOLOGICA

THE JOURNAL OF CLINICAL CYTOLOGY

Vol. 15 January February 1971 No 1

CONTENTS

An Invitational Symposium

Automated Cytology

Chapter I Objectives of Automated Cell Analysis

Chapter II Classical Quantitative Measures of Cell Properties *George L. Wied and Gunter F Bahr (Editors) Departments of Obstetrics and Gynecology and Pathology University of Chicago Chicago Illinois and Biophysics Branch, Armed Forces Institute of Pathology Washington, D C U S A* 1

Articles

Aspiration Biopsy of Tumors of the Neck. II Cytologic Findings in 13 Cases of Carotid Body Tumor *U Engzell S Franzen and J Zajicek Department of Otolaryngology Radium-hemmet and Department of Clinical Cytology Institute of Tumorpathology Karolinska Sjukhuset Stockholm Sweden* 25

Cytologic Diagnosis of Renal Cell Carcinoma with the Aid of Fat Stain. *Steven I Hajdu, Angela Savino Eva O Hajdu and Leopold G Koss Cytology Service Department of Pathology Memorial Hospital for Cancer and Allied Diseases New York, New York and Division of Laboratories and Research, Nassau County Department of Health Hempstead New York, U S A.* 31

New York State Computerized Proficiency Testing Program in Exfoliative Cytology Development. *Doris N Collins and William Kaufmann. Division of Laboratories and Research New York State Department of Health, Albany New York, U S A.* 34

The Chromosomal Aberration of Double Minutes in a Human Embryonic Rhabdomyosarcoma *Ingrid Granberg and Joachim Mark. Cytodiagnostic Department University Hospital and Institute of Pathology and Institute of Genetics University of Lund, Lund, Sweden* 42

Continued on next page

Malignancy Associated Changes in Buccal Smears. *Robert R. Finch and Emmerich von Haam. Department of Oral Pathology, University of Louisville School of Dentistry, Louisville, Kentucky, and Department of Pathology, Ohio State University College of Medicine, Columbus, Ohio, U.S.A.* 46

Quantitative Criteria of Folate Deficiency in Cervico-Vaginal Cytograms with Report of a New Parameter. *Hanna Klaus. Department of Obstetrics and Gynecology, Washington University School of Medicine, St. Louis, Missouri, U.S.A.* 50

Quality Control in Cytology. A Proposed Method for Selection of Slides for Review. *Bernard Klionsky and Martin Cohen. Department of Pathology, Magee Womens Hospital, and Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, U.S.A.* 54

Vulvar Cytology. *R. Tozzini, A. J. Sobrero and E. Hoovis. Margaret Sanger Research Bureau, Inc., New York, New York, U.S.A.* 57

Fellows and Members of the International Academy of Cytology 64

Editorial Notice 71

News from Societies of Cytology 72

The European Federation of Cytology Societies 72

Asociacion Mexicana de Citologia Exfoliativa 72

Sociedade Latino-americana de Citology 72

Sociedad Latinoamericana de Citologia Ejercicio 1966–1970 73

Sociedad Argentina de Citologia 77

British Society of Clinical Cytology 77

Ceskoslovenská Společnost pro Gynekologickou Cytologii 77

Österreichische Gesellschaft für Zytologie 78

Svensk Förening for Klinisk Cytologi 78

Sociedad Espanola de Citologia 79

Nippon Rinsho Saibo Gakkai 80

Finnish Society for Clinical Cytology 80

Canadian Society of Cytology 81

American Society of Cytology 82

Joint Meeting of the Swiss, German and Austrian Societies of Cytology 83

Announcements 84

Fourth Tutorial on Clinical Cytology (Chicago) August, 1971 84

Mount Sinai School of Medicine 84

ACTA CYTOLOGICA

THE JOURNAL OF CLINICAL CYTOLOGY

Vol 15 March April 1971 No 2

CONTENTS

Letter to the Editors

Histochemical Study of Hydroquinone Tetrazolium Reductase Reaction in Plasma Cells. *F Hernández and J R Iglesias Department of Histopathology University of Navarra Pamplona Spain* 85

An Invitational Symposium continued

Automated Cytology

Chapter III Other Parameters Descriptors to be Considered 87

Chapter IV Descriptor Definition and Measures of Discriminatory Power

George L Wied and Gunter F Bahr (Editors) Departments of Obstetrics and Gynecology and Pathology University of Chicago Chicago Illinois and Biophysics Branch, Armed Forces Institute of Pathology Washington, D C U S A 116

Articles

The Imprint Method for the Cytodiagnosis of Lymphadenopathies and of Tumors of the Head and Neck. *Rudolf Aust Jan Stahle and Bjorn Stenkvist Department of Otorhinolaryngology and the Department of Clinical Cytology University Hospital Uppsala, Sweden.* 123

Morphology of Exfoliated Cells in Benign Gastric Ulcer *Joao C Prolla, Rogerio G Xavier and Joseph B Kirsner Department of Medicine University of Chicago Chicago Illinois U.S.A.* 128

The Cytologic Diagnosis of Tissue Repair in the Female Genital Tract. *Marluce Bibbo Catherine M Keebler and George L Wied Departments of Obstetrics and Gynecology and Pathology University of Chicago Chicago Illinois U.S.A.* 133

Automatic Detection and Classification of Cells in Cervical Smears *J H Tucker Department of Pathology University of Cambridge Cambridge England, U K* 138

Malignancy Associated Changes in Buccal Smears. *Robert R. Finch and Emmerich von Haam Department of Oral Pathology University of Louisville School of Dentistry Louisville Kentucky and Department of Pathology Ohio State University College of Medicine Columbus Ohio U S A* 46

Quantitative Criteria of Folate Deficiency in Cervico-Vaginal Cytograms with Report of a New Parameter *Hanna Klaus Department of Obstetrics and Gynecology Washington University School of Medicine St Louis Missouri U S A* 50

Quality Control in Cytology A Proposed Method for Selection of Slides for Review *Bernard Klionsky and Martin Cohen Department of Pathology Magee-Womens Hospital and Department of Pathology University of Pittsburgh School of Medicine Pittsburgh Pennsylvania, U S A* 54

Vulvar Cytology *R Tozzini A J Sobrero and E Hoovis Margaret Sanger Research Bureau, Inc New York, New York U S A.* 57

Fellows and Members of the International Academy of Cytology 64

Editorial Notice 71

News from Societies of Cytology 72

The European Federation of Cytology Societies 72

Asociacion Mexicana de Citologia Exfoliativa 72

Sociedade Latino-americana de Citology 72

Sociedad Latinoamericana de Citologia Ejercicio 1966–1970 73

Sociedad Argentina de Citologia 77

British Society of Clinical Cytology 77

Ceskoslovenská Spolecnost pro Gynekologickou Cytologii 77

Österreichische Gesellschaft für Zytologie 78

Svensk Förening for Klinisk Cytologi 78

Sociedad Espanola de Citologia 79

Nippon Rinsho Saibo Gakkai 80

Finnish Society for Clinical Cytology 80

Canadian Society of Cytology 81

American Society of Cytology 82

Joint Meeting of the Swiss German and Austrian Societies of Cytology 83

Announcements 84

Fourth Tutorial on Clinical Cytology (Chicago) August, 1971 84

Mount Sinai School of Medicine 84

ACTA CYTOLOGICA

THE JOURNAL OF CLINICAL CYTOLOGY

Vol 15 May June 1971 No 3

CONTENTS

Letters to the Editors

Transthoracic Needle Biopsy for Diagnosis of Lung Tumors. *Patricia Alonso Hector Ponce de Leon and Fernando Cano-Valle Laboratory of Cytopathology and Pneumology Service Hospital General S S A Mexico City Mexico.* 209

Atypical Cells in Spermatocele Fluid *Paul Wentworth Sharon Wagar and Mary Unitt Clinical Laboratories Brantford General Hospital Brantford, Ontario Canada* 210

The Etiology of the Cocci Type 'Streptokokkentyp Vaginal Smear *Pentti A Leppaluoto Laboratory of Cancer Society Tampere Finland* 211

An Invitational Symposium

Automated Cytology (continued)

Chapter V Requirements of Preparation and Sample Collection Problems 216

Chapter VI Approaches to Prescreening 236

Articles

Cell Recognition by Multivariate Gray Value Analysis in Digitized Images *Peter H Bartels Jack M Layton Thadeus L. Jarkowski John C Bellamy Gunter F Bahr and George L. Wied Department of Microbiology and Optical Sciences Center University of Arizona Tucson, Arizona Department of Pathology University of Arizona School of Medicine Tucson Arizona Department of Pathology Tucson Medical Center Tucson Arizona Biophysics Branch Armed Forces Institute of Pathology Washington, D C Departments of Obstetrics and Gynecology and Pathology University of Chicago Chicago Illinois U S A* 284

Machine Characterization of Human Leukocytes by Acridine Orange Fluorescence. *Laurence R. Adams and Louis A Kamentsky Bio/Physics Systems Inc. Mahopac New York, U.S A* 289

Continued on next page

Computer Recognition of Cells from Asymptomatic Lymphocytic Leukemia I Methodologic Study *Thaddeus L. Jarkowski Jack M Layton Gunter F Bahr George L Wied John C Bellamy and Peter H Bartels Department of Pathology Tucson Medical Center Tucson Arizona and Department of Pathology University of Arizona College of Medicine Tucson, Arizona et al U S A* 147

Tumor Diagnosis in Pleural and Ascitic Effusions Based on DNA Cytophotometry *S C Freni J James and F J A Prop Department of Pathology and Histological Laboratory University of Amsterdam Amsterdam Holland* 154

Ultrasonic Dispersal of Cervical Cell Aggregates *Janet S Parry Brendan K Cleary A Roy Williams and David M D Evans Department of Cytology Research Group Laboratory University Hospital of Wales (Cardiff) H M C St David's Hospital Cardiff and Department of Microbiology University College of South Wales and Monmouthshire University of Wales Cardiff Wales U K* 163

A Comparative Study of the Estrogenic Potential of Two Synthetic Estrogens (Mestranol and Ethinylestradiol) *Jerzy Teter and Romuald Stupnicki Department of Clinical Endocrinology Medical Academy in Warsaw and Department of Endocrinology Institute of Animal Physiology and Nutrition, Polish Academy of Sciences Jablonna Poland* 167

Preliminary Report on the Incidence of Cervical Carcinoma in Situ in Iraq *Karama Kubba Medical Research Centre University of Baghdad Baghdad Iraq* 171

Chromosomal Findings in a Case of Leukemic Reticuloendotheliosis *Alessandra Forni Mario Baroni and Emma Pacifico Clinica del Lavoro L. Devoto' University of Milan Milan, Italy* 173

Primary Malignant Melanoma of the Vagina A Case Report. *C Milton Linthicum Physicians Service Laboratory Towson Maryland, U S A* 179

Abstracts from the First Symposium on Cytology

Presented at the First Meeting of the European Federation of Cytology Societies 182

Book Reviews 196

News from Societies of Cytology 197

Announcements 201

ACTA CYTOLOGICA

THE JOURNAL OF CLINICAL CYTOLOGY

Vol. 15 May June 1971 No 3

CONTENTS

Letters to the Editors

Transthoracic Needle Biopsy for Diagnosis of Lung Tumors. *Patricia Alonso Hector Ponce de Leon and Fernando Cano-Valle Laboratory of Cytopathology and Pneumology Service Hospital General S S A Mexico City Mexico* 209

Atypical Cells in Spermatocele Fluid. *Paul Wentworth Sharon Wagar and Mary Unitt Clinical Laboratories Brantford General Hospital Brantford, Ontario Canada.* 210

The Etiology of the Cocci Type Streptokokkentyp Vaginal Smear *Pentti A Leppaluoto Laboratory of Cancer Society Tampere Finland* 211

An Invitational Symposium

Automated Cytology (continued)

Chapter V Requirements of Preparation and Sample Collection Problems. 216

Chapter VI Approaches to Prescreening 236

Articles

Cell Recognition by Multivariate Gray Value Analysis in Digitized Images *Peter H Bartels Jack M Layton Thadeus L Jarkowski John C Bellamy Gunter F Bahr and George L Wied. Department of Microbiology and Optical Sciences Center University of Arizona, Tucson, Arizona Department of Pathology University of Arizona School of Medicine Tucson, Arizona Department of Pathology Tucson Medical Center Tucson Arizona Biophysics Branch, Armed Forces Institute of Pathology Washington, D C Departments of Obstetrics and Gynecology and Pathology University of Chicago Chicago Illinois U.S A* 284

Machine Characterization of Human Leukocytes by Acridine Orange Fluorescence. *Lawrence R Adams and Louis A Kamentsky Bio/Physics Systems Inc Mahopac New York U.S A* 289

Continued on next page

The Incidence of False Negative Cervical Cytology as Determined by Colposcopically Directed Biopsies *Bernard Gondos Department of Obstetrics and Gynecology and Department of Pathology Harbor General Hospital Torrance California and University of California School of Medicine Los Angeles California U S A* 290

Comparison of X Chromatin Frequency in Buccal and Vaginal Cells from Atopic Women *Eloise W Kailin and Lois I Platt Department of Medicine Howard University School of Medicine Washington D C and Department of Pathology George Washington University Washington D C U S A* 294

In Vitro Observations of Sex Chromatin in Cells from the Human Uterine Cervix. *George D Wilbanks and James A Campbell Department of Obstetrics and Gynecology Oncology Division, Duke University Medical Center Durham, North Carolina U S A* 29

Comet or 'Decoy' Cells Found in Urinary Sediment Smears *J G S Crabbe Cytology Training Centre Royal Free Hospital London W C 1 England U K* 303

Peritoneal Fluid Cytology Associated with a Solid Ovarian Teratoma in a Mouse *Larry McGowan Robert H Davis and Bill Bunnag Department of Obstetrics and Gynecology George Washington University Medical Center Washington, D C and Department of Obstetrics and Gynecology Hahnemann Medical College Philadelphia Pennsylvania U S A* 306

Mycoplasma and Vaginal Cytology *P A Mårdh N Stormby and L Westrom Institute of Medical Microbiology University of Lund Department of Cytodiagnostics University Hospital and Department of Obstetrics and Gynaecology University Hospital Lund Sweden.* 310

Simple Method for Prenatal Determination of Sex. *J H Arendsen and H J Huisjes Department of Obstetrics and Gynecology University Hospital Groningen The Netherlands* 316

W H O Nomenclature in Cytology 318

Book Review 320

News From Societies 321

ACTA CYTOLOGICA

THE JOURNAL OF CLINICAL CYTOLOGY

Vol 15 July August 1971 No 4

CONTENTS

Letters to the Editors

On Barr Bodies and Cell Senescence. *Gunter F Bahr Biophysics Branch, Armed Forces Institute of Pathology Washington, D C U S.A* 325

Effect of 3-Isothiazolone on Human Cancer Cells *Donald W McKinstry William L. West Sidney Green, Wilfred D Crow and Nelson J Leonard College of Medicine Howard University Washington, D C and Department of Chemistry and Chemical Engineering University of Illinois Urbana Illinois U.S.A* 325

Proceedings of the 18th Annual Scientific Meeting of the American Society of Cytology Los Angeles, California, November 1970

Presidential Address. *Robert H Fennell Department of Pathology Presbyterian-University Hospital of the University Health Center of Pittsburgh, Pittsburgh, Pennsylvania U S A.* 327

Comparison of Hormonal Colpocytology with Endometrial Histology in Gynecologic Patients. *J Victor Reyniak, Alexander Sedlis Dorothy Stone and Poungpen Rimdusit Department of Obstetrics and Gynecology New York Medical College-Metropolitan Hospital Center New York, New York, U S A* 329

Clinicopathologic Studies in Feminizing Tumors of the Ovary III The Role of Genital Cytology *William W Johnston, William R Goldston and Martha S Montgomery Departments of Pathology and Obstetrics and Gynecology Duke University Medical Center Durham North Carolina, U.S A.* 334

Exfoliative Cytology of Primary and Metastatic Wilms Tumors. *Steven I Hajdu. Cytology Service Department of Pathology Memorial Hospital for Cancer and Allied Diseases New York, New York, U.S A.* 339

Preparation of Cytologic Smears from Solid Tumors. *Leslie Anne Norquist and Abraham I Schweid. Cytology Laboratory Department of Pathology University of Washington-Harborview Medical Center Seattle Washington, U.S.A* 343

Careers in Cytotechnology—Seventeen Years Experience. *Richard D Otis Anne L. Griswold and Jean M Nauss School of Cytotechnology Department of Pathology Hartford Hospital Hartford Connecticut U.S A.* 347

Continued on next page

A State Wide Proficiency Testing Program in Cytology *Stanley L. Inhorn and Edna Clarke Wisconsin State Laboratory of Hygiene University of Wisconsin Madison Wisconsin U.S.A*
351

Atypical Cells in Pleural Fluid Characteristic of Systemic Lupus Erythematosus *Susan Kelley Paul McGarry and Yolande Hulson Louisiana State University Medical Center and Charity Hospital of Louisiana New Orleans Louisiana U.S.A*
357

Observations of Eosinophilic Extracytoplasmic Processes in Pulmonary Macrophages Progress Report. *Kenneth R. Walker and Cyril D Fullmer Department of Cytology Holy Cross Hospital Salt Lake City Utah U.S.A*
1

Light and Electron Microscopic Observations on Malignant Cells in Cerebrospinal Fluid from Metastatis Alveolar Cell Carcinoma. *William W Johnston Fred L. Ginn and Joseph M Amatulli Department of Pathology Duke University Medical Center Durham North Carolina U.S.A*
36

The Evaluation of Sex Chromatin as a Prognostic Factor in Carcinoma of the Breast. A Preliminary Report. *Angela Sanino and Leopold G Koss Cytology Service Department of Pathology Memorial Hospital for Cancer and Allied Diseases New York, New York U.S.A*
365

Further Experience with Direct Vision Brushing Cytology of Malignant Tumors of Upper Gastrointestinal Tract Histopathologic Correlation with Biopsy *Joao C Prolla, Yuri Yoshii Rogerio G Xavier and Joseph B Kirsner Department of Medicine University of Chicago Chicago Illinois U.S.A.*
372

Squamous Cell Changes Associated with the Presence of *Candida sp* in Cervical Vaginal Papanicolaou Smears *Carol Heller and Virginia Hoyt St Cabrini Hospital and Harborview Medical Center Seattle Washington U.S.A*
375

Exfoliative Cytology of the Urinary Bladder Irrigation Specimen *M J Harris C P Schwinn J W Morrow R L. Gray and B M Browell Departments of Surgical Pathology and Urology Los Angeles County-University of Southern California Medical Center Los Angeles California U.S.A*
379

Cytologic Differentiation of Carcinoma of the Breast by Electron Microscopy *Tariq M Murad Department of Pathology College of Medicine Ohio State University Columbus Ohio U.S.A.*
385

Circulating Megakaryocytes A Quantitative Observation in Normal Subjects by Age and Sex. *Carla Dago Charles M Karpas Louis Pincus Alex Tytun and Abraham Oppenheim Department of Pathology Beekman-Downtown Hospital New York, New York U.S.A*
400
410

Announcements

The International Academy of Cytology 414
The European Federation of Cytology Societies 416
Canadian Society of Cytology 417
Cours de Cytologie Vaginale Hormonale 417

ACTA CYTOLOGICA

THE JOURNAL OF CLINICAL CYTOLOGY

Vol 15 September October 1971 No 5

CONTENTS

Letters to the Editors

Antioxidant Inhibition of Stain Fading and Mounting Medium Crazing *David H Hollander and John K Frost Division of Cytopathology Department of Pathology Johns Hopkins University School of Medicine Baltimore Maryland U S A* 419

The Use of Crushing Condenser for Photometric and Other Cytologic Studies on Histologic Sections *N B Atkin Department of Cancer Research Mount Vernon Hospital Northwood Middlesex England, U K* 419

Invitational Symposium on Automated Cytology

Chapter VII Sorting or Marking the Prescreened Cells 422

Chapter VIII Patterns of Cell Populations 444

Articles

The Quinacrine Fluorescence Method of Y-Chromosome Identification. *David H Hollander and Digamber S Borgaonkar Division of Cytopathology Department of Pathology and Division of Medical Genetics Department of Medicine Johns Hopkins University School of Medicine Baltimore Maryland U.S A* 452

Comparative Sequential Cytologic Changes Following in Vitro Infection with Herpesvirus Types I and II *R L. Teplitz Z Valco and T Rundall Department of Cytogenetics City of Hope National Medical Center Duarte California U S A* 455

Early Diagnosis of Small Peripheral Lung Cancer—Cytologic Diagnosis of Very Fresh Cancer Cells Obtained by the TV Brushing Technique. *Shoji Hattori Minoru Matsuda Hiromu Nishihara and Takeshi Horai Department of Lung Cancer and Division of Diagnostic Cytology Center for Adult Diseases Osaka Japan.* 460

Continued on next page

New York State Computerized Proficiency Testing Program in Exfoliative Cytology Evaluation. *Doris V Collins William Kaufmann and Robert Albrecht Division of Laboratories and Research New York State Department of Health Albany New York U S A* 46

Calcified Bodies and the Intrauterine Device. *Wilma J Highman Department of Cytology St George s Hospital Tooting London, England U K* 47

Findings of Microfilarial Larval Stages in Gynecologic Smears *Rosa de Borges Cytology Section Institute of Pathology Hospital Vargas Caracas Venezuela* 47

A Cytologic Study of Dipetolenema Perstans in Cervical Smears *Santosh D Sharma Olin Zeigler and Richard R Trussell Department of Obstetrics and Gynaecology (Cytology Section) Makerere University Medical School Kampala Uganda* 47

Evidence of Gynecologic Bilharziasis in Cytologic Material A Morphologic Study for Cytologists in Particular *Ann Berry Cytology Unit Department of Pathology South African Institute for Medical Research Johannesburg South Africa* 48

Cytochemical Determinations of Dry Mass and Protein Content and Cytometry in Bull Spermatozoa *Gunter F Bahr and George L Wied Biophysics Branch Armed Forces Institute of Pathology and Department of Pathology Georgetown University Washington D C and Departments of Obstetrics and Gynecology and of Pathology University of Chicago Chicago Illinois U S A* 49

Cytology of Ileal Conduit Urine *Richard A Malmgren Mark S Soloway Elizabeth W Chu Pasco R DelVecchio and Alfred S Ketcham National Cancer Institute National Institutes of Health Bethesda Maryland U S A* 50

nnouncements

Fifth Tutorial on Clinical Cytology 510

Registry Examinations for Cytotechnologists C T (I A C) Registry 510

Ecole Superieure de Technique Anatomo et Cyto-Pathologique 511

ACTA CYTOLOGICA

THE JOURNAL OF CLINICAL CYTOLOGY

Vol. 15 November December 1971 No 6

CONTENTS

Articles

Tissue and Cell Pathology of Uterine Cervix Dysplasias and Carcinoma in Situ *Herbert E Nieburgs Department of Pathology Mount Sinai School of Medicine of City University of New York, New York New York U.S.A* 513

A Test Statistic for Detecting the Presence of Abnormal Cells in a Sample. *Prodyot F Bhattacharya, Peter H Bartels Gunter F Bahr and George L. Wied Departments of Mathematics and of Microbiology and the Optical Sciences Center University of Arizona Tucson Arizona Biophysics Branch Armed Forces Institute of Pathology Washington D C Departments of Obstetrics and Gynecology and of Pathology University of Chicago Chicago Illinois U.S A* 533

Transformation of Mononuclear Cells in Cerebrospinal Fluid. *Rune Sornas Department of Neurology University Hospital Lund Sweden.* 545

A Classification of Nuclear Aberration in Relation to Malignancy Associated Changes (MAC) *Robert R Finch, Health Sciences Center School of Dentistry University of Louisville Louisville Kentucky U S A* 553

DNA Values from Cytology in a Case of Palatal Carcinoma *P Pfitzer and D H Pape Pathologisches Institut und Klinik für Kiefer und Gesichtschirurgie der Universität Dusseldorf Düsseldorf Germany* 559

Forum on Criteria for Certification, Licensure and Quality Control of Cytology Laboratories. *Held during the re-convened 18th Annual Scientific Meeting of the American Society of Cytology in Honolulu, Hawaii* 562

Erratum 577

Book Reviews 578

Continued on next page

Announcements

Norsk Forening for Klinisk Cytologi (Norwegian Society of Clinical Cytology) 579

Indian Academy of Cytologists 579

Sociedade Latino-Americana de Citologia 580

Canadian Society of Cytology 580

Sociedad Argentina de Cytologia 580

Fifth Tutorial on Clinical Cytology 581

Subject Index for Volume 15 583

Author Index for Volume 15 586

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AUTOMATED CYTOLOGY

A Symposium by Correspondence

PREFACE

Editors

George L. Wied, M.D., F.I.A.C. and Gunter F. Bahr, M.D., F.I.A.C.

The following Symposium by Correspondence on Automated Cytology was conducted to discuss the newest developments of techniques, hardware, software and the general approaches to automation in cytologic diagnosis and screening.

The Symposium is too voluminous to be published in one issue of the Journal and will appear in several issues throughout the year 1971. Some of the topics of the Symposium are still under review and discussion, so that the reader may be assured of up-to-date information on this matter of great concern to the cytopathologist and cytotechnologist.

The participants in the Symposium are listed separately on the onset of each chapter. The individual chapter titles will be as follows:

Part A

I Objectives of Automated Cell Analyses and Separation
II Classical Quantitative Measures of Cell Properties
III Other Parameter Descriptions to be Considered
IV Descriptor Definition and Measures of Discriminatory Power
V Requirements of Preparation and Sample Collection Problems
    a) For Screening Procedures
    b) For Targeted Analyses of Single Cells
VI Approaches to Prescreening
VII Sorting or Marking the Prescreened Cells
VIII Patterns of Cell Populations

Part B

IX The Targeted Analysis of Single Cells and Tissue Areas by Optical Pattern Recognition (including self-learning and self-optimizing routines)
X Features of a Fully Integrated System
XI Systems Designs
    a) On-line Local Data Processing
    b) Time-Sharing at a Central Facility
    c) Hybrid Concepts
XII Machine Specifications, Confidence Limits
XIII Cost Effectiveness Considerations
XIV Impact on Training of Personnel and Staffing of Automated Laboratories
XV The Medico-Legal Situation

Chapter I Objectives of Automated Cell Analysis

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Opening Remarks

Drs Bahr, Bartels and Wied

In discussing automated analyses of cells, a clear understanding of its present diversity of objectives must be gained which are deceivingly united by the adjective "automated."

Grossly we distinguish two major essentially different objectives. One is directed towards the increase in *quantity* of the cytopathologic diagnostic product. The other seeks to improve the *quality* of information derived from a cytologic sample.

There is no doubt that existing laboratories are, in most instances, able to cope with the case load they receive from hospitals and practicing physicians. As soon as true population screening projects come underway i e as soon as there is a significant increase in the number of cases to be handled, the low incidence of interesting cases will put a tiresome burden on human screeners which are eliminating the negatives. Ensuing decreased alertness and attrition of motivation will surely be attended by increased turnover of personnel and lessened significance of the test.

A machine *out-performing* the job of the screener—not of the qualified cytotechnologist—would indeed open the gate to mass screening It is not surprising, therefore that the medical profession, governments, and industry have taken intense interest in developing a machine that would be as ubiquitous as cytology laboratories.

A machine of whatever type must fulfill some basic premises before it can be accepted

a) It must recognize an inadequate sample, e g one in which only leukocytes occur or no cells at all
b) It must screen out better than 90 *per cent* of the normals with no significant errors, otherwise one would have to keep a most experienced staff negating machine errors on cytologic material degraded by the analytic process, which would be an expensive proposition Machine reliability should be such that no smears have to be kept, except a full regular assessment of the cellular material seems to be indicated The collection of test material must remain simple machine

must work for appreciably less cost per case than the human screener not only to become economically interesting, but most of all because it produces so much less information about the specimen in simple yes-no decisions. This also means that the machine procedure has to be significantly faster than one or several screeners.

) It must have minimum periods of down-time, because we cannot afford pile-up of unprocessed samples. There will not be screening staff anymore, waiting in the ready to take over again. One should not be forced either to buy and keep back-up machine.

d) Unique sample identification is required in a flow-through system that processes closely spaced samples, which is difficult because cells are not only stickier than chemicals, but they also have different sizes and move, therefore, at different speeds. No sample mix-up can be permitted.

It is my considered opinion that no machine whatsoever is in sight fulfilling these requirements now nor in the near future. Interested parties are cautioned not to relent on requirements for more and better training of cytotechnologists and cytopathologists.

Serious efforts currently underway in a few laboratories will eventually produce machines for screening of cytologic and also of hematologic material. First there will be prototypes. Finally finished products will be offered. The readers of this symposium are reminded to proceed with caution not only in procuring an instrument, but more so with respect to the premature release of trained staff. Only full field test, carried out in the frame of the special conditions that often prevail in different laboratories, paralleled and compared to regular screening procedures by trained personnel can provide an answer to the usefulness and economy of new device. Until such time, fully operating screening machine is not closer but also no farther away than a human landing on Mars.

Another totally different objective of automated cell analysis, unfortunately often confused with the above discussed, is improvement of the quality of our diagnostic produce. A priori and intuitively we understand that improvement of quality can only be concerned with areas of cytology and hematology in which we encounter difficulties in making clinically satisfying decisions, the so-called grey areas or areas of continued debate. We find that the cellular images involved do not seem to render enough unambiguous clues to the physiology or pathology of the condition.

It is, therefore, the foremost aim of this qualita-

experienced eye and the best of microscopically reenforced vision. Most importantly it should be noted that evaluation of each cell image is done objectively, is on record, is repeatable, and that classical laws of probability are automatically applied to inform the cytopathologist about the confidence limits of the information.

Fortunately this avowed qualitative aim is not subject to the stringent requirements of the quantitative objective. Only cells of interest are selected from visual field. No normals have to be analyzed and rejected. Time of analysis is of secondary importance as long as the diagnostic product is unique, i cannot be obtained by any other method now known and serves a clinical purpose. Only few cells are necessary for this approach. Functioning machinery already exists, is sturdy and does not currently exceed the cost of an electron microscope.

Once enough qualitative machines are around (by comparison there are now over 10,000 electron microscopes functioning in the world) one might think of other useful areas of application, for which such instruments were not originally designed. These would be

) Unsupervised teaching of basic cytology and hematology. A student would submit a cell image from his slide (without removing it from the microscope) to the computer and receive confirming or rejecting answer to his simultaneously transmitted identification.

b) Long distance consultation of the type being practiced in the United States for electrocardiograms and clinical chemistry data. A series of cells, difficult to classify by less experienced cytologist or hematologist, working at a remote hospital may be submitted by telephone to large, adequately programmed computation center as reduced cell data for identification. Data reduction would have been accomplished by a small, relatively inexpensive computer ($2,000–$4 000) working on-line to the microscope.

c) At some distance in time we see the application of computer assisted scanning to histologic material also.

There are many other uses to which cellular analytic systems of this sort could be employed, not the least of which would be a hook-up to a well functioning cell screening and sorting machine, taking over the last steps of identifying types of cells separated or marked as not normal."

DR. MELLAMED

Automation in the medical laboratory is desirable if it increases the capacity to perform tests veloped and very successful. For others, including many of the examinations of cellular specimens, individual study by skilled technicians is still essential.

It may be that the complex judgments required for certain cellular examinations preclude full automation, but there is good reason to believe that automatic devices can and will be of substantial help. Satisfactory techniques are already available for counting the total number of cells in a specimen, and for determining volume distribution of cell populations.[2] [3] Identification and enumeration of component cells in cell mixtures is considerably more difficult, but there is sound theoretical basis for believing that automated systems of cell classification also can be developed.[4] Advances in computer technology and electronics, and new light sources, promise to simplify many of the design problems.

It is appropriate at this time to consider the objectives of an automated system for cell analysis that would be of use in the clinical and research laboratory. Specific requirements must eventually be governed by the types of cells to be studied and the nature of the examinations, but a preliminary statement of general goals should be made now. These may be listed from simplest to more complex.

I Identification of specimens as normal or abnormal

The most fundamental kind of differentiation is the distinction between normal and abnormal, and it is an appropriate goal for the first automated instruments. Many, perhaps most, of the cellular specimens submitted to the medical laboratory are normal. In the case of cytologic examinations for uterine cervical carcinoma, over 99 per cent will come from women without cancer. If all normal specimens, or a large proportion of them, can be recognized as such by an automated instrument, the full attention of technical personnel can be devoted to specimens that are abnormal. This would make more efficient use of the cytotechnologist, who in turn would have more time for examining cases of special interest.

II Separating, concentrating or marking cells according to predetermined criteria

The first step in assessing certain specimens could be by combining human judgment with a device for concentrating or marking all abnormal or unusual cells in the specimen. Such a device would not make value judgments about the abnormality but judgments could be made by examination of the cells selected. Very often the abnormal cells in a specimen are few and considerable time may be spent searching for them manually. By selecting the cells that require study the time spent on each specimen could be lessened.

Devices that physically separate or concentrate the abnormal cells also provide a mechanism for obtaining uniform cell populations, and facilitate examination by other modalities, e.g. biochemically, comparing with morphology.

III Recognition of specific cells or cellular components

Cytochemical stains, radio-isotopes or antigen-antibody complexes can be used as labels for specific cells or cellular components. Automated instruments that would search for and recognize these "labels" could be used to identify particular cells. For example, granulocytic leukocytes in a specimen of blood might be identified by stains for peroxidase or alkaline phosphatase, certain epithelial cells by their mucin content, the presence of keratin, glycogen or specific secretory products. Fluorescent labelled antibodies could be used to identify cells immunologically, and might be a means of screening specimens for pathogenic bacteria or parasites.

Quantitative differences in cellular components or in staining characteristics also can be used to identify certain kinds of cells. Epidermoid cancer cells, for example, characteristically contain more deoxyribonucleic acid than their benign counterpart. Eventually, very fine distinctions between cells may be possible by distributional differences in intracellular components.

IV Characterizing and displaying the distribution of cell types

Classification and enumeration of the cells in a mixed cell population is the ultimate goal for automated analysis of cellular specimens, and it will require highly sophisticated instrumentation. At present, classification is based on cell morphology, but there is no reason why it could not be based on cell function or cellular constituents, or some other factor or combination of factors. The differential white blood cell count is a tempting model for this kind of analysis, and it has been used with instruments that show some promise of success.

V Providing computational facilities and data banks for evaluation and interpretation

By applying interactive computer based processes, data from appropriate measurements may be helpful in classifying problem cells.[1]

VI Providing unique methods to characterize cells

Instrumentation offers a broad potential range of parameters for examining cells, exceeding that possible with the ordinary light microscope and it is likely that unique methods for characterizing cells will be developed. These may use ultraviolet or other non-visible energy for making measurements. They may assess cell function, chemical constituents of the cell or its organelles, or some kind of cellular activity.

Design Requirements

In order to attain the objectives outlined above an effective instrument must satisfy the following requirements

I Cells must be individually examined.

It is essential for all types of cellular specimens that each cell in representative sample be individually examined Measurements of total cell populations that yield average values, and do not indicate the variations of individual cells, are useless for discriminating between classes of cells in mixed cell populations or for detecting the presence of few abnormal or unusual cells

II Measurements must correlate with light microscopy

At present, classification of human cells is based upon their appearance by light microscopy Cell types or cell abnormality as defined by any new instrument must correlate with cell types or cell abnormality as defined by light microscopy though the measurements themselves may be made using other techniques For example, cell size can be determined by using the Coulter principle of electrical conductance which yields measurements that agree very well with cell size determinations by light microscopy

III Results must be displayed in usable manner

The method of displaying results must suit the objective of the examination. In the case of cancer detection cytology specimens, for example, the display must quickly draw attention to any abnormal cells even though they may be very few For classification and enumeration of blood cells, the relative proportion of major cell types is much more important

IV Results must be reproducible

Aliquot samples of any specimen must yield identical results In fact, major advantage of examination by automated instrumentation is the desirability of obtaining identical results in different laboratories or t different times with the same specimen. Ideally also, the cells should not be lost or destroyed by the examination, and should be re-usable later for the same or other tests

V Cells must be examined at rapid rate

Individual cell examinations must be sufficiently rapid so that the thousands of cells normally present in most specimens can be examined within reasonable period of time It should be possible to process umber of specimens in less time than would be required manually

VI Cell collection must remain simple

The method of collecting specimens must remain simple for the physician or technician, and it must provide representative material Preparati methods must maintain cellular integrity and accentuate cell separation or dispersion to facilitate measurements of individual cells. The method of preparation and medium in which cells are examined must not adversely ffect cellular characterization In fact, it should enhance characteristic differences

Future Goals

As the first automated instrument systems for cell analysis are developed, there will be modifications and perhaps major changes in the objectives outlined here. At the least, requirements will become much more specific. It is possible that families of instruments will be necessary each tailored to particular test. Fundamentally different principles may be used for different instruments according to the examinations they must perform, or several different measuring systems may be incorporated in one instrument. Computers will surely be necessary for data storage and evaluation, and perhaps to govern the function of automated instruments

What will finally be the role of utomation in examining and evaluating cellular specimens cannot be guessed t present. The objectives outlined at this time represent only the starting point of what promises to be a long and exciting chapter in the story of laboratory medicine.

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DISCUSSION

DR. FULWYLER

My purpose here is to discuss the objectives of automated cytology and two opening papers on this topic the first, by Myron R. Melamed is veloped and very successful. For others, including many of the examinations of cellular specimens, individual study by skilled technicians is still essential.

It may be that the complex judgements required for certain cellular examinations preclude full automation, but there is good reason to believe that automatic devices can and will be of substantial help. Satisfactory techniques are already available for counting the total number of cells in a specimen, and for determining volume distribution of cell populations.[2, 3] Identification and enumeration of component cells in cell mixtures is considerably more difficult, but there is sound theoretical basis for believing that automated systems of cell classification also can be developed.[4, 5] Advances in computer technology and electronics, and new light sources, promise to simplify many of the design problems.

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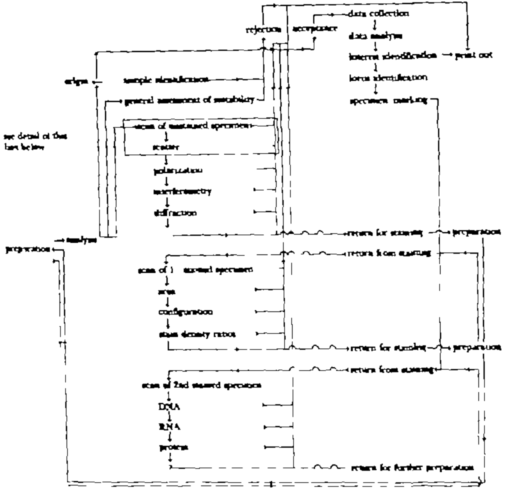
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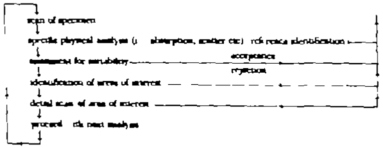
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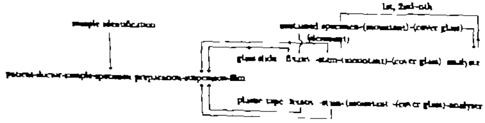
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entitled "Objectives of Automated Cell Analysis" and the second, by Gunter F Bahr with the same title. In his excellent paper Dr Melamed has very clearly outlined the objectives and requirements of automated cytology. Because I find myself in agreement with his views, I will not comment further but will go on to the second paper

In his paper Dr Bahr distinguishes two essentially different objectives, one of which is directed toward increasing the number of cytological samples which can be processed and the second of which seeks to improve the quality of diagnosis. The impression is given that these goals are incompatible. Further he assumes that any prescreening instrument will be capable of only a yes-no decision on each cell giving no more than enumeration of the "normal and abnormal" cells. This is a very limited view of the potential capabilities of high-speed sensors of bulk cell properties.

Our group is developing a method of automated analysis in which several properties of each cell will be measured *quantitatively* and rapidly (i.e in 100 μ-sec) Signals produced by each sensor and representing a specific cell property will be processed singly or in combination and the resulting information stored. After analysis of an entire sample of about 50 000 cells, distribution histograms of each single property or of selected combinations of properties will be formed and made available to describe and characterize the total cell population. This type of data (i.e. distributions of properties among large numbers of cells) is very different in character from the information given by analyzing a few cells and classifying each in one of several categories. Analysis of large numbers of cells yields data (distribution curves) of high statistical precision.

Such precision makes it possible to discern small shifts in the distribution which may reveal pathologic changes before they are of such a magnitude as to render single cells clearly abnormal. This does not exclude the ability to make simple normal-abnormal decisions on each cell but, in fact, gives complementary information which will aid interpretation of cells classified as abnormal. As Dr Bahr points out, no prescreening machine exists or is in sight. Although some of the benefits or limitations of such devices can be envisioned many cannot and I feel that Dr Bahr has erred in his prejudgment of their capabilities.

An example of analysis of the distribution of a property among large cell populations is found in the work of Anderson and Petersen in which they studied the dependence of growth and division on cell age and volume.[1] This type of rapid instrumental analysis, yielding data of high statistical precision, is quite new to the fields of biology and medicine but would seem to hold great potential for producing new fundamental knowledge of cell systems.

One final point the orientation of this symposium is to apply new techniques to old problems such as the Papanicolaou test or the white blood cell differential count. Perhaps we should enlarge our thinking to include the broader objective of obtaining new quantitative information on properties of individual cells and on the distribution of these properties among various cell systems. In accepting such broad long-range goals, we are motivated to undertake study of cell properties which are now or can be made measurable and to study how they are related to one another and to cell function and physiological state. As these relationships become known, specific instrumentation requirements and fruitful clinical applications will become apparent. This approach may be a more efficient way in which to exploit the potential of automated cytology and could reveal quite unanticipated applications. Perhaps this should be our goal.

REFERENCE

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DR HALE

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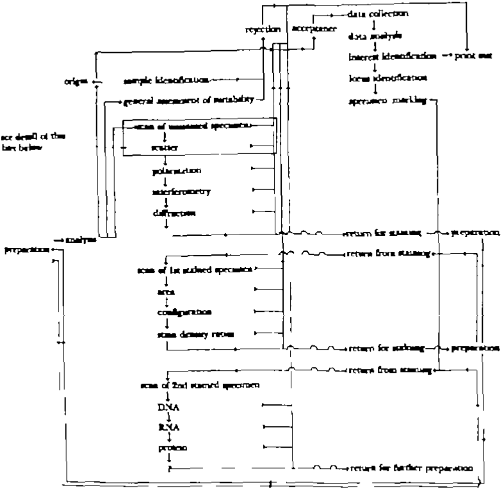
I agree with the comments on the need for multiparameter analysis and evaluation of *new* diagnostic criteria which correlate with the clinical status and *not* the cytopathologists opinion. This is probably the most difficult psychological hurdle to be surmounted by the cytopathologist but if it is not overcome all such analyses will still rest on comparison with subjective personal diagnostic opinion.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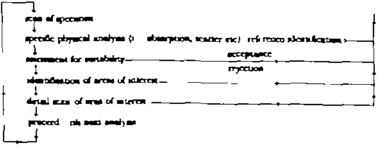
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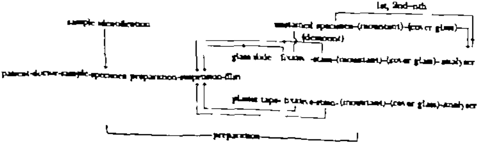
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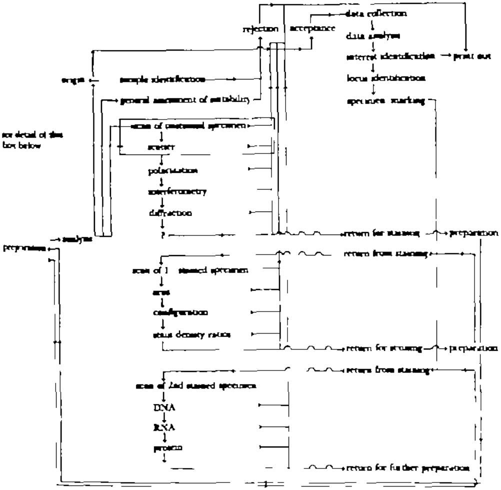
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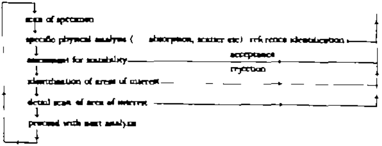
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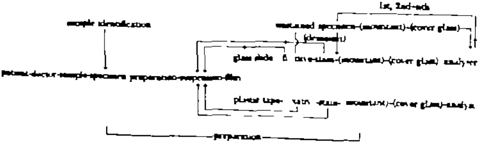
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1 Anderson, E. C. and Petersen, D F Cell growth and division. II Experimental studies of cell volume distributions in mammalian suspension cultures. Biophys. J 7: 353 364 1967

Dr. Hale

Much has been said in these papers and elsewhere about the difficulties associated with automated cell analysis but I cannot remember seeing anywhere an analysis and grading of the difficulties. An analysis would serve to draw attention to the factors which limit the development of such apparatus and, thus, might concentrate *attention on the critical factors* For *example* an expert on pattern recognition might have an excellent solution to that factor in the overall problem but totally ignore or only partially solve the problem of specimen presentation. It would seem logical to use a critical path analysis to identify the important factors Scheme I is an attempt to do so but in my experience this path *must* be commented on and *altered* by other discussants if it is to end up as a truly critical analysis.

I agree with the comments on the need for multiparameter analysis and evaluation of *novel* diagnostic criteria which correlate with the clinical status and *not* the cytopathologists opinion. This is probably the most difficult psychological hurdle to be surmounted by the cytopathologist but if it is not overcome all such analyses will *still rest* on comparison with subjective personal diagnostic opinion.

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DR. SAUNDERS

With reference to characterizing cells, any other than morphologic means will require a massive re-education process for the user. Functional cytology will hold many surprises, not the least being the variability of response between young and old cells of very similar morphology. Hence new bases for characterizing cells will have to develop along with automation.

DR. SPRIGGS

The replacement of men by machines is not an end in itself. Machines are made and sold to meet economic demands, either for work that cannot be done without them, or to improve quality or accuracy, or to give a better output per unit cost. The possibility that people may buy a machine for purposes of prestige will not be considered here.

First it is worth mentioning three non-objectives of automated cell analysis and separation, so far as they relate to cervical cells.

1. The screening of cervical smears can be done by specially trained people with a degree of reliability which is usually considered adequate, provided that smears are repeated at intervals throughout adult life. The possibility of a machine *improving* on the accuracy of a cytotechnician is an interesting subject of speculation. At the moment, human performance is the yardstick against which any machine has to be measured, and an improvement would be quite difficult to detect or measure even if it could be attained. For a machine to match the human performance, it is necessary to decide on criteria by which to fix the dividing lines between positive, dubious and negative. There is a possibility of using criteria which cannot be detected by the human eye—for instance UV absorption—but the more usual ones known to be of discriminating value such as size, irregularity and staining intensity of nuclei are exactly those noted by the human observer. Now if positive smears could be separated from negative ones simply by a combination of a few criteria of this sort, whether automated or not, why is it that it takes about three months before a human can learn the difference? The same human could learn to recognize all the current models of cars or airplanes in a day or two, even a foreign language can be mastered for simple purposes in less time than it takes to recognize malignant cells. Let us then admit that positive smears cannot be distinguished reliably by simple criteria, but only by very complex ones which are not consciously analysed. At the moment it would be folly to pour out money on efforts to match the human performance.
2. In discussions on cytology automation, the possibility is often raised of discovering some new measurable parameter which is characteristic of malignant cells. Those who make this suggestion do not always realize that the search for specific characters of malignant cells has been pursued in vain for many decades, by workers who had no thought of automation or even of applications to cervical smear diagnosis. It is a large and baffling area of cancer research into which we must not, with our specific aim, allow ourselves to be diverted.
3. A third non-objective in my view is the recording of high-resolution scanning data on individual cells for diagnostic purposes, as for instance "long-distance consultation," or the compilation of banks representing expert opinions of different cytologists on various cell types, or for unsupervised teaching as suggested by Bahr. All these suggestions depend on the notion that a cell can be identified in isolation, that a meaningful label can be attached simply from a series of measurements. This is not what an experienced diagnostic cytologist does. He makes a subconscious provisional identification based on (a) the characters of the cell in question, (b) the characters of other cells in the vicinity, and (c) his knowledge of the source of the material and of variations in the way in which it was collected and prepared. On looking at other parts of the slide his initial assessment may change, and the diagnosis is derived after seeing many thousands, sometimes even a million cells. Another reason for placing long-distance consultation among the non-objectives is that, in the case of vaginal or cervical smears, the smear is made for detection and not for final diagnosis. Nobody wants the opinions of distant (or defunct) experts on difficult individual cells. If the whole slide is difficult, then a biopsy will settle the question.

Having specified three targets which are not, in my opinion, those to which we should aim, what are then our reasonable first objectives? There seem to be two, both perhaps capable of realization in the foreseeable future. They are the first two enumerated by Melamed in this discussion.

1. *A division, not the final one, into negatives and a set containing the positives.* This type of approach

Dr. Husain

Professor Bahr's account is very much to the point and I would like to comment on one or two aspects.

He states that machine reliability should be such that no smears have to be kept. We have been faced with this problem in the development of the Quantimet Image Analyzing Computer[4] with its latest edition, the Cytoscreen, which is now undergoing trials. If the philosophy is to keep the initial pick up or alerting cell sample along with all the subsequent confirmatory and follow up smears then obviously an automated slide smear sample is preferable to the cell tape preparation being developed by us and other workers. However, as Professor Bahr states, the processed cell sample has lost much of its diagnostic criteria during cell dispersal and this may *not* be the best example to be retained.

It also follows that, at least for some time to come, the detection system will require that a repeat or confirmatory test should be by the most effective sampling and screening techniques to make the important diagnosis of cancer. This is without question the cervical or endocervical scrape or swab scanned by experienced eye.

One could then conclude that tape transport—with its much greater facility for automation, and freedom from the manual handling of the individual slide smears with its staining, mounting, trimming, labelling, sorting and filing—is the method of choice. The greatest advantage of the glass slide and coverslip is its optical clarity and the interchangeability with orthodox microscopy. The signalling of a raster scan on slide is easily coded on the unsmeared label end of the slide and *this could be used on any microscope with a* simple decoding device attachment. Nevertheless, the ultimate for speed and handling must be tape transport.

The greatest argument for automation is the one of removal of the intolerable boredom of repetitive tests with a 'pick up' rate down in the 2 to 5 per 10 000 range (incidence rate). The danger of being at the mercy of one machine and its likelihood of breakdown means, as with all automated systems, that there should be more than one in any unit, and therefore it is assumed that large screening centers are to be the mode.

I would endorse the caution Professor Bahr gives to the impulse buyers of automated equipment in this field who anticipate automation eclipsing the technician and that there is no need to continue cytologist training schemes. This will not be so for many years to come, if ever at all. In our philosophy the automated seeker-scanner will require even better trained cytoscreeners than hitherto, as they will have to assess on fewer fields, and there will be need for almost as many workers as at present as the service will then expand to screen effectively the whole population.

The one instrument we are concerned with, the Quantimet, and now its new purpose-built successor, the Cytoscreen, are both very much research machines and there is no suggestion of building a trial service machine at this stage.

The objectives of automated cell analysis as instanced by Dr Melamed define the most *essential, yet simple aspects of the problem.* The failure of such instruments as the Cytoanalyzer in the past was not only because of inadequate electronics but due to the faulty premise that an instrument could recognise a whole cell sample as abnormal or containing abnormal cells—of which there may be very few—rather than the present more cautious and defined approach of identifying the abnormal cell. This, by means of separators such as Fulwyler's, or by side stream on to membrane filter by the Kamentsky technique, or even by the scatter graphs of Kamentsky's spectrophotometric oscilloscope demonstrates that even a few cells with high nuclear cytoplasmic ratios could be separately registered.

The basic aim is, of course, to separate the abnormal from the normal cell sample in order that attention can be paid to the former. If this can be achieved by a form of general pattern or matrix analysis or by a simple enzyme estimation such as the 6 phosphogluconate dehydrogenase within the cell, then the final course is open to either cytologist confirmation or a superimposed analytic scanner to finally identify the malignant cell.

As Dr Melamed says, this may be achieved on an elaborate matrix analysis or by the intricate parameter checking system such as *TICAS* (Taxonomic Intra-Cellular Analytic System) or a combination of techniques, a sort of test profile utilizing enzymic chemical pattern recognition, fluorescent and functional test. An example of this approach could be the use of simple fixed cell scanners of cell tracks on film strip to identify the carcinomas-in-situ and dysplastic cells which are large and darkly-staining, then at menopause the biochemical 6 PGD test is added to this test sample in order to detect the vaginal glandular cases.

The enzymes are always raised in invasive disease whereas the scanners can easily miss the faintly-staining or minute or degenerate almost unrecognizable endometrial cancer cell.

That cell collection should be cheap and simple is essential. The collection of a scrape smear is the most expensive part of the test and the use of aspirate or irrigation smears should be considered, especially because of the convenience and cheapness of collection and also because the cells are essentially in suspension—and therefore more *amenable to automated scanning*—and *not buried* in clusters.

stitute an important area for analysis. Similarly the non-cellular elements in a tissue preparation e.g abundance or otherwise of collagen fibers, may likewise be fruitful to explore.

In addition, the objective of automatic cell analysis may not only be related to screening procedures where the obviously abnormal cell is selected for analysis The detection of significant deviations, such as those indicating malignancy in its earliest phase is of clinical importance as are those which indicate other types of altered physiologic state. To meet these requirements necessitates a high order of sophistication in the analytic approach adopted

With regard to the design requirements needed in order to achieve the objectives already discussed several points are worth mentioning First, and in many instances, we are trying to replace subjective impression by one which we feel is more in agreement with the objective physical world. Not knowing how the human eye and mind achieves their analyses of objects we attempt recognition of cell differences by machine To accomplish this satisfactorily and in the most expeditious fashion often involves a "trade-off" between what might be considered theoretically desirable and what is practically achievable

In the last analysis the automatic analysis of cells only has validity and purpose in terms of the practicality of its outcome

REFERENCE

1 Papanicolaou, G N Atlas of Exfoliative Cytology Cambridge Massachusetts, Harvard University Press, 1963

DR VASTOLA

Some additional comments regarding leukocyte differential counts seem appropriate Each hospital bed in this country generates between 100 and 200 differential counts per bed per year some of the variability is explained by variations in the amount of associated outpatient work and by the character of the hospital's academic affiliations At Kings County Hospital (2,200 beds) in Brooklyn the figure is 250 000 at Barnes Hospital (1020 beds) in St Louis 150,000 and at the New England Medical Center Hospital (400 beds) in Boston 70,000 One hundred cells, the customary number can be counted in five minutes but the average time over a normal working day is about twice as long The work is tedious and the results, in many instances unreliable The low motivation and rapid turn-over of poorly trained laboratory technicians in the typical city hospital are no more easily seen and their consequences more familiar than in the clinical hematology division. In most city hospitals these counts, unless performed by the medical student or house officer do not make a significant contribution to the clinicians' decision-making processes If the count confirms a judgment based on other data, the clinician is reassured, if not, it is disregarded. From a laboratory service of this kind, information regarding immature forms, toxic granules, alterations induced by viral illnesses in lymphocytes and monocytes, etc cannot be expected. An automated system would have to provide such capabilities, as well as recognition of the five normal adult forms, count between 200 and 400 cells in 2 to 4 minutes and utilize existing methods of preparation, supplemented, perhaps, by a five to one leukocyte enrichment process. Unlike the situation in regard to exfoliative cytology it is probable that blood cytology does not offer a field of substantial usefulness for an operation limited to a screening function If rapid screening were the goal it would have to be based on the neutrophil count other measures, with present technology require the recognition of traditional patterns of color and structure an art, which, if available, could constitute, without much more extensive application, a complete and definitive classification scheme However it is estimated that 25 to 35 per cent of the abnormal specimens obtained in a general hospital would not be identified as such by an examination limited to the total leukocyte count and the percentage of neutrophils In the case of outpatients, the proportion of abnormals missed by such an examination would probably be higher In a multiphasic screening clinic, the proportion missed would be even higher A system that provided satisfactory classification of leukocytes could also process red cells for morphology at little additional cost.

CLOSING REMARKS

Dr Bahr

For the engineer clinician, pathologist or biologist interested in the automation of cell analysis, and especially cell identification with clinical aims, the preceding discussion not only reflects the magnitude of the problem but also some significant diversity of view More important than any difference in opinion is the fact of notable consent on many of the prerequisites and conditions an automated system must fulfill before it can be accepted in a clinical setting or as a useful tool in related biological research

I agree with Dr Fulwyler that research applications of automatic cell identifiers and/or sorters to tissue cultures will probably precede application to vastly more complex clinical material. My skepticism is primarily addressing itself to prospects of handling the latter material

Dr Husain's attention to the boredom of cytotechnicians in mass screening projects is well taken.

could be helpful in saving time, but if the machine used material prepared in some way other than conventional smears then the latter would need to be made at the same time so that patients do not have to be called back.

Possibilities for this approach would be biochemical tests (e.g. for enzymes), colorimetric methods with or without the use of dyes, (particularly for DNA content per nucleus), labelling with radio-active isotopes, and methods of cell sizing in liquid suspension.

Supposing that a test of this kind was able to reject without much false negative error 80 per cent of those samples would be negative by conventional examination, or come from cervices with no significant lesions. At first one might imagine that this method, suitably automated, would be of great value. But this would only be the case if the same imperfect and partial separation could not be done more economically by humans. We all know the expertise necessary for a proper examination of a cervical smear. But a relatively untrained and low-paid worker, spending only a minute or less on each smear, might well be able to match the performance of our postulated sorting machine. A second rate machine should be compared, for economic effectiveness, with second rate screeners, and a deliberate evaluation of the latter perhaps deserves to be made at this point as part of the research effort on automation.

2. *Automated marking of fields containing suspicious cells, or actual separation and concentration of abnormal cells.* The currently favored parameters for marking are nuclear diameter and *staining density*, and if large dark nuclei can be reliably pinpointed on a smear this can hardly fail to save time in scanning because less than the usual number of fields need to be looked at. But the same remarks apply to this system as to the last, in that the performance of such a machine should be compared with that of a human individual *trained only to do what the machine does.* Screeners asked only to mark large dark nuclei would certainly do the work more accurately than any foreseeable *machine*, because they could unconsciously reject all sorts of artefacts which are not single nuclei at all. It does not seem too ridiculous to give such work to screeners with hardly any training, perhaps working on this for short periods each day; the cells which they marked would be examined by cytotechnologists, who would *look at all marked* cells, and examine the whole of every smear containing even one marked cell which, to a trained eye, seemed genuinely aberrant. The choice between this, nonautomated, system and the development *of a machine can be made here and now* because the first is immediately available, while the second is at a developmental stage and without the certainty of being finally successful. The main objection to the use of human labor in this way is the problem of status and payment for semi-skilled workers at the laboratory bench.

Separation and concentration of abnormal cells *by mechanical means* would, *if successful*, represent a great advance. Clearly the technical problems are formidable, probably more than for "marking" systems. Conventional preparations would need to be available as well, and the interpretation of the concentrates would probably require a new and different expertise. In principle, the value of concentration of relevant material, as with "*marking*" *systems, depends* on the time saved, and there will be no advantage if the same labor is needed to work and maintain the apparatus.

Dr. Steward

Dr. Melamed's description of objectives in automated cell analysis admirably covers many aspects of the problem. Also as indicated by him the objectives outlined at this time can only perhaps represent a beginning. However, in the preliminary statement of general goals he lists the identification of normality or abnormality of a specimen as the simplest out of a number which are to be attained. Rightly he describes this as the most fundamental type of differentiation one can make. But in this discussant's view such an endeavor can also be the most complex. The validity *of all subsequent analyses are dependent upon it.* Further, it should be remembered that the decision process by which cells may be separated into distinct classes is one that ultimately involves human judgment; this includes the setting up of arbitrary standards with which comparisons can be made.

Also, the question of normal versus abnormal need not rest entirely on the analysis of individual cells as stated by the author. Thus, as with humans, the biological significance of cells may be indicated by the company they keep. For instance, malignancy in cervical smears may in part be indicated by an abnormal aggregation or grouping of cells, and in many tissues disordered cellular patterns and parenchymal debris may give a first clue as to deviations from normal. Thus in tissue sections derangement of cell patterns, e.g. those occurring in the cerebral cortex and those *forming blood-vessels*, may con titute an important area for analysis. Similarly the non-cellular elements in tissue preparation g abundance or otherwise of collagen fibers, may likewise be fruitful to explore.

In addition, the objective of automatic cell analysis may not only be related to screening procedures where the obviously abnormal cell is selected for analysis. The detection of significant deviations, such as those indicating malignancy in its earliest phase is of clinical importance, as are those which indicate other types of altered physiologic state. To meet these requirements necessitates high order of sophistication in the analytic approach adopted.

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Dr Spriggs doubts the possibility of improving human visual diagnostic capabilities I believe that the history of medicine is determined to a large extent by such improvements and have no doubt that pattern recognition techniques will be able to refine cytodiagnostic criteria and solve diagnostic difficulties on a routine basis

Pattern recognition has taken the place of the human eye and ear in analysing electrocardiograms and heart sound For the unexperienced this can be done over the telephone instead of using the mail

Since single cells will be identified as is now a tank or a missile by pattern recognition in aerial photography from satellites, there is no reason to reject the concept of transmitting a cell's image or any image for that matter by telephone or satellite in its original or a reduced form. Dr Spriggs is correct in stating the unique capabilities of the human eye but when one looks at the million cells he mentions one is likely to have lost the engram of the first while looking at the last A computer memory will not forget.

The next chapters of this Symposium will tell us how many of the stated requirements can be fulfilled now in the future or maybe never

Dr Spriggs is pointing out a most relevant fact, which is only too often passed over with silence and this is the relatively long training period required for humans to recognize a tumor cell for what it is Furthermore Dr Spriggs as a highly experienced cytopathologist, confirms that the diagnostic decision is made on the basis of complex, and not consciously analyzed criteria The example chosen by Dr Spriggs is a good one different types of automobiles, or airplanes are learned quickly I would like to point out here, though, that these objects represent "good patterns in the sense that few if any alternative classification decisions would be possible A decision about the model of a car is made with likelihoods of approximately 10^4 1 Tumor cells, and particularly well differentiated ones are definitely not good patterns. Classification decisions about them are made with likelihoods closer to 10 1 and in rare instances 10^3 1

I quite agree with Dr Spriggs that naive hopes to find a simple measure present in, and unique for all tumor cells, are most likely to lead to disappointment I would not agree that attempts in this direction are but a diversion Computation, and EDP technology have opened avenues of approach previously simply not feasible There is something different in the texture and appearance of the tumor cell which makes visual recognition finally possible The search for quantitation, and extraction of this quality may involve complex analysis of the stochastic nature of the images, but it seems to me to be a valid course of investigation. Some of our presently employed multivariate gray value analysis programs recognize lymphocytes in different physiologic states with likelihoods in the order of magnitude of 10^3 1 when visual observation is unable to make a clear cut distinction.

As far as Dr Spriggs evaluation of the usefulness of objective image assessment is concerned, I believe that there is a misinterpretation of purpose on his part. A clinical sample from a patient is a course to be evaluated in its entirety and a diagnostic decision made on the basis of a sample drawn from the entire cell population on the slide The composition of this sample is defined, however by the diagnostic decisions made for individual cells, which represents a lower level of diagnostic decision making and this has been pointed out by us early It remains necessary to diagnose even a single cell with high reliability When a machine can do this with very high likelihood on the basis of known, and well defined image criteria, I would not call this worthless

I am quite optimistic that meaningful labels can indeed be attached to single cells, although they may not be derived from a few simple measurement

Dr Spriggs points out that an expert should revise his opinions constantly on the basis of new incoming information Central data banks with constant updating and preserved information will certainly be the technologic means of doing just this, and here in lies the value of objective image assessment Photographic recordings cannot be readily transmitted updated, computed and adapted or be used to form a data base for statistics about criteria which one is searching for It is precisely this sort of information which might lead to development of an effective recognition procedure the adequacy of which would be known before it were ever implemented by actual hardware This is why I do not share Dr Spriggs opinion that cell image objectivation for diagnostic purposes is a non-objective for research

Dr Melamed

I have read the discussions of my paper and Dr Bahr's papers on "Objectives of Automated Cell Analyses" and feel that closing remarks from me would be unnecessary

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Chapter II Classical Quantitative Measures of Cell Properties

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Opening Remarks

Drs. Sandritter and Kiefer

Most prominent among the quantitative measures of cell properties are quantitative histochemical and cytochemical methods microphotometry cytophotometry) in the ultraviolet and visible regions of the electromagnetic spectrum, microinterferometry, microfluorometry and x-ray-historadiography. These methods have gained great importance in general cell biology. Some findings obtained by these methods have contributed to the characterization of tumor cells and revealed differences between cancer and normal cells. (Autoradiography and other cytotopochemical methods as, for instance, enzyme histochemistry play a rather subordinate role in this respect.)

Microphotometry is predominantly used in the visible spectral region for the determination of DNA content of single cells. For this purpose DNA is stained by the Feulgen nuclear reaction. This staining reaction shows an unsurpassed specificity for this cell compound. For quantitative purposes the specimen must be stained under conditions where stochiometric relationships hold, i.e. the amount of the bound dye must be proportional to the amount of DNA present in the cell. As the proportionality factor between DNA

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I am quite optimistic that meaningful labels can indeed be attached to single cells, although they may not be derived from a few simple measurement

Dr Spriggs points out that an expert should revise his opinions constantly on the basis of new incoming information. Central data banks with constant updating and preserved information will certainly be the technologic mean of doing just this, and herein lies the value of objective image assessment. Photographic recordings cannot be readily transmitted updated, computed and adapted, or be used to form a data base for statistics about criteria which one is searching for It is precisely this sort of information which might lead to development of an effective recognition procedure the adequacy of which would be known before it were ever implemented by actual hardware This is why I do not share Dr Spriggs opinion that cell image objectivation for diagnostic purposes is a non-objective for research

Dr Melamed

I have read the discussions of my paper and Dr Bahr's papers on "Objectives of Automated Cell Analyses" and feel that closing remarks from me would be unnecessary

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See EDITORIAL NOTICE p. 71

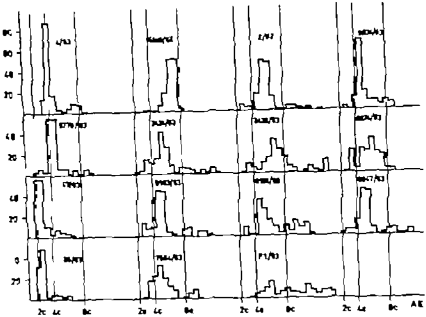

FIG. 3 DNA Feulgen histograms from different tumors. 2c, 4c, 8c = diploid, tetraploid and octoploid DNA values, respectively. DNA is measured in arbitrary units (AE). (from *Sandritter et al.* 1966)

volved in DNA replication (G cells) exist whose DNA content deviates from that of normal diploid or tetraploid (euploid) nuclei. This deviation is parallel to the changes in chromosome number (aneuploidy). Corresponding to the chromosome *number stem line concept* this DNA content characterizing given tumor cell population has been called DNA stem line.

DNA *measurements from several hundred* cases of cancer have been reported and in every case significant deviations of DNA content from euploid values could be *observed*.

The most frequent values were found to be slightly lower than tetraploid (hypotetraploid). During invasive *growth, changes of stem line* DNA content could be observed in few cases of cancer of the cervix uteri. By cytophotometric DNA measurements it is therefore possible to determine whether suspicious cells (cancer?) are present in population or sample of biologic material. The usefulness of cytophotometry for diagnostic purposes is substantially limited by the fact that normal cells in the proliferating phase can be present in the sample. These cells have an increased DNA content, too, and would lead to "false positive" results. This obstacle can be partially overcome by measuring great number of cells. In this case the histogram of proliferating normal cells is superimposed on that of the cells with deviating DNA stem line. These cells *can therefore* be detected as second peak in the histogram, if they occur in sufficient frequency within the sample (Fig. 3).

There exist number of approaches for using DNA content as diagnostic criterion. Specifically modified microphotometers have been used which enable measurements on several thousand cells within few minutes or even seconds. With this instrumentation, cells are not placed on a microscopic slide, but are moved as suspension in capillaries in front of the microscopic optics. The use of fluorescent Feulgen dyes enable more accurate DNA determination in these flow photometers.

Until now no wide-scale screening experiments have been reported. *Definitive* judgment of the usefulness and limitations of such methods is therefore not yet possible.

Caspersson, by *UV* absorption studies, observed high cytoplasmic RNA concentration in the cells of invading tumors. The high RNA content is *responsible for the increased* basophilia of the majority of cancer cells. A few basic dyes, for example, gallocyanine chromalum, stain both RNA and DNA *in stochiometric* proportion and allow cytophotometric evaluation of total nucleic acid content. Studies on gallocyanine chromalum stained vaginal *smears* have shown that smears from cases classified as Papanicolaou Classes IV or V contain much higher proportion (up to 84 per cent) of cells with *an elevated* nucleic acid content than cells from unsuspicious cases (Papani-

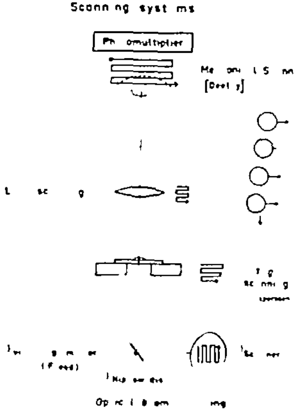

Fig. 1 Schematic representation of different scanning systems.

and dye amount is unknown, DNA content is expressed in arbitrary units (AU) In general slides to be compared are fixed and stained *together to assure identical conditions* The reliability of the reaction has been intensively studied Only small deviations from proportionality occur in cells with highly condensed chromatin[12, 7]

Microphotometric techniques originated from the early work of Caspersson[7, 8] Caspersson eliminated the error introduced by inhomogenous distribution of material within the cell by scanning the microscopic image of the specimen with a small aperture. The principle of this method has been retained in the most modern cytophotometers, while the technical solution of the scanning mechanism can be different (Fig 1) A second method to avoid distributional error is the so-called two wavelength method.[10, 11]

Microphotometric measurements of Feulgen stained nuclei have led to the establishment of the DNA constancy concept. According to this hypothesis the DNA content is the same within all nuclei of an organism and is independent of metabolism and activity of the cell[30] Only three exceptions from this rule are generally accepted (1) polyploid or polytene cells, (2) proliferating tissues (3) cancer cells. Some rare cases show minor alterations of the species specific DNA amount per nucleus e g "DNA puffs" of salivary gland chromosomes of some dipters[14] or in "amplificated" cistrons for ribosomal RNA in the oocytes of newts, water beetles and crickets[4] Whether DNA content increases in all activated genoms is doubted.[1, 21, 22] In every case a mechanism of this kind would lead to an insignificant rise of the total amount of DNA per nucleus. Because of this relative DNA constancy the variability of measuring values from a population of diploid non-proliferating cells is small and can be attributed to errors of the techniques used[11, 21] If there are cells with a higher ploidy present, the measuring values are grouped around mean values which differ by a geometric progression 2 4 8, etc (Fig 2) In a growing cell population only cells in the G_1 phase exhibit the DNA content characteristics for diploid cells. Cells in the G_2 period of interphase possess two times this amount, while cells in the S-phase show intermediate values. By this the frequency distribution (histogram) of DNA values from a proliferating cell population shows a characteristic shape (Fig 2) From the relative frequency of the cells in G_1, S, and G_2 phases, respectively the proliferation kinetics of the population can be calculated.[20, 31]

Deviation from DNA constancy found or in tumor cell populations are quite different from those just described A majority of cell nuclei not in-

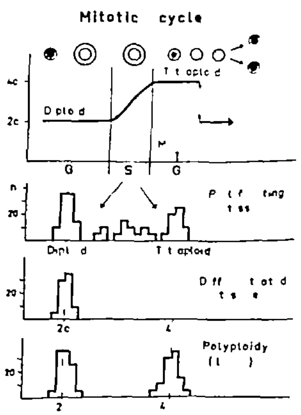

Fig 2. Mitotic cycle and DNA distribution patterns of different tissues.

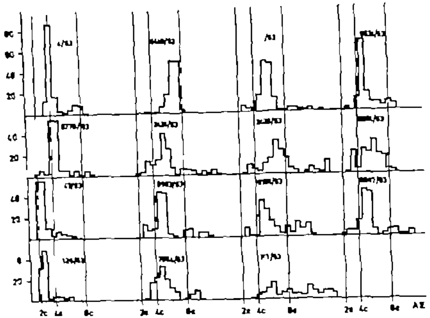

FIG. 3 DNA-Feulgen histograms from different tumors. 2c, 4c, 8c = diploid, tetraploid and octoploid DNA values, respectively DNA is measured in arbitrary units (AE). (from *Sandritter et al.* 1966)

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Interferometric total dry mass measurements have only been used for the characterization of tumor cells in a few cases.[9, 26] This method might be a tool with regard to the fact that instrumentation is available which allows large numbers of measurements within a reasonable time.[15] As a basis for investigations of this kind a thorough analysis of dry weight determinations on cells from normal vaginal epithelium is available.[26]

Future studies in the field of the quantitative cytochemistry of tumors should pay attention to possible correlation between different parameters (DNA, RNA, *protein, dry mass*) *within the same* cell to get further insight into the nature of cancer

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DISCUSSION

DRS. BARTELS, BAHR AND WIED

Dr. Sandritter has given us a comprehensive survey of research in the area of nucleic acid cytophotometry. The numerous literature citations all refer to papers in which tumor cells were shown to have DNA values markedly different from the values found in normal cells.

It would seem that before DNA content could be used as a single parameter to assess cell normal or abnormal condition, a concerted effort would have to be made to collect a comparable data base for a wide variety of clinical conditions. Using standardized measuring procedures, and rigidly maintained staining conditions the mean squares for the patient to patient, slide to slide, staining batch to staining batch, laboratory to laboratory variance components would have to be determined to estimate the true standard of error of the technique.

This would enter into the confidence limits, which would also form the basis for estimates of the probability of errors of the first and the second kind. This data base does not presently seem to exist in a centrally collected form, but it would seem feasible to set up standards and to establish such a data bank.

Dr. Sandritter himself, however, raises the question of whether Feulgen reaction cytophotometry would really lend itself as an exclusive parameter for the problem at hand. In a prescreening situation, one is not trying to distinguish a sample of cells with normal DNA complements from a sample of cells having abnormal nuclei.

In the clinical situation the sample is likely to contain a small percentage of abnormal cells. The majority of cells has DNA values corresponding exactly to the distribution found in a normal cell sample—which might well contain a few outliers with abnormally high values.

Could Dr. Sandritter, on the basis of his experience, comment on what kind of a test statistic would be useful to make the decision: number of outliers does not exceed value expected for a normal sample versus too many outliers, presence of abnormal cells likely?

What likelihood ratios for the correct recognition of a tumor cell versus a normal cell can one expect in Feulgen stained material, or in material stained with Gallocyanin? What mean differences can we expect in the high value wing of the Feulgen value distribution for normal and abnormal samples, and what relative variances of the relative frequencies of occurrence? If one assumed a 99 per cent confidence level, could Dr. Sandritter on the basis of material measured by him, give us an estimate on the discriminatory potential of the technique?

DR. HALE

Absorption microspectrophotometry and microinterferometry are the two techniques which have been most intensively used in quantitative analysis of single cells. Investigations have provided data which correlate well with studies using other techniques, e.g. aneuploid amounts of DNA in cell nuclei of certain tumors corresponding to the change in karyotype in these tumors. In addition, data has been produced showing that increases which may be attributable to tumors in one set of conditions are reproduced by non-malignant cells under another set of physiological conditions, e.g. the accumulation of protein in adrenal nuclei obtained from adrenals which have undergone hypertrophy.

It is also clearly the case that identification of a few cells with a peculiar DNA or other value is of no great significance since there are very few occasions where this aberrant value does not fall within the theoretical normal range. A pattern of distribution at first sight does help (see Atkin & Richards, 1959) but it is not sufficiently positive to permit a diagnostic decision on that criterion alone.

Analyses of this type have laid the foundation for objective quantitative cellular analysis but the problem is much like trying to identify an automobile from the kind of tires which it uses. The criteria are insufficient to permit decisions.

DR. HYMAN

The account by Professors Sandritter and Kiefer regarding the quantitative measures of DNA colaou Classes I and II).[27] Measurements of total nucleic acid content using a UV microphotometer also showed higher nucleic acid content of cancer cells.[9]

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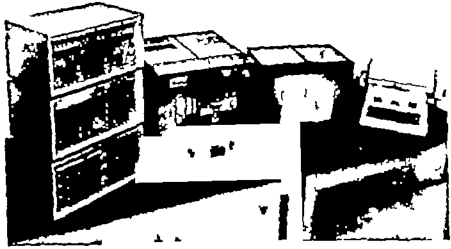

FIG. 4. Metals Research Quantimet B

ing, in this Symposium, it might be impossible for machine to substitute human screeners' ability the present status of engineering level, but our prototype apparatus Photoenderoscanner" may contribute to the quantitative evaluation of classic cytodiagnostic parameters

DR MAWDESLEY THOMAS, MESSRS HEALEY AND BARRY

It is unfortunate that whilst the need to quantify optical images has long been realised, so much attention has been focussed on producing methods and machines which will differentiate the malignant cell This in itself is an unenviable task, for the morphologist cannot accurately define the criteria of malignancy for himself, much less for machine Machine recognition of tumor cells can only be of limited value in screening procedures as human emotive forces will never permit a diagnosis of malignancy on purely mechanical grounds There are however many other fields in which quantitative methods can be used with competence

One of the most recent advances of significance in image analysing computing is the Metals Research "Quantimet B" (Fig. 4) This instrument has since 1968, been used with great success in the assessment of experimental bronchitis in rats, * pulmonary emphysema, altered hematopoietic activity of bone marrow measurement of thyroid colloid, and alteration in size of pancreatic islets associated with various physiologic and pathologic conditions The Quantimet B uses the principle of television scanning and will measure area size and count particles, cellular details, etc which can be shown up in sufficient optical contrast to their background[4]. This machine has recently been superseded by an improved model, the Quantimet 720 produced by Image Analysing Computers Limited, subsidiary of Metals Research Limited, Royston, Cambridge, England, who pioneered the initial machine. The 720 (Fig 5) being digital in concept, has many advantages over the old model B. These analysers can scan an image in a fraction of second enabling statistically significant area of tissue or cells to be counted and or measured in short period of time (1 2,000 fields/hour) The uses of this type of analysis are unlimited and could well alter the more traditional concepts of cell pathology

The desirability of short term screening tests of potential carcinogenic agents is obvious. This need is paramount in tobacco condensate testing where innumerable tar fractions from marketed or experimental tobaccos are evaluated for tumorigenic activity The measurement of sebaceous gland suppression is one such test[5] The original test never acquired the popularity it deserved, due to technical difficulties. The modifications of this technique involve serial frozen sections of mouse skin and the histochemical demonstration of non-specific esterase enzyme in sebaceous glands. The non-specific esterase activity is shown and RNA in cells in various stages of replication, be they of a proliferative or malignant nature, does demonstrate the possibility of a concise assessment of this relatively exact parameter. Exact in the way of its measurement rather than of content, as Atkins and others have pointed out.

Feulgen and gallocyanine chromatin stains are exact techniques and provide satisfactory measures of DNA and RNA in ideal conditions. They are difficult to harness to the raw unselected cell samples with background mucoproteinaceous and bacterial exudates. However the authors have managed successfully to utilize the fluorescent Feulgen technique for rapid photometric techniques in creating both the characteristic histogram and a possible separation of the euphoidic from the non-euphoidic groups. It depends on the source of the cell sample as to whether it will have a small or large fraction of abnormal cell types. For instance an endocervical swab sample would contain a ratio of 110 100 abnormal/normal cells, a cervical scrape smear 35 100 and the vaginal aspirate only 2-3 100 abnormal/normal cells.[1] A histogram may not suffice to present a deviating cell type in some cell samples. This is especially so in the face of proliferating non-malignant tissue.

The register of accurate DNA content of nuclei by whatever cytophotometric method used must be accurate to within ±2 per cent and be of reasonable rapidity to sort out a cell sample. Something of the speed of their fluorescent flow photometers would be ideal.

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Dr Ishiyama

In this discussion, I would like to introduce our thoughts on automated cytodiagnosis based on geometry and densitometry of microscopic images. Our research group intends to automate data-acquisition and data processing of "classical quantitative measures" for diagnostic prescreening. The reason we have adopted classical quantitative measures is described in Chapter VI "Approaches to Prescreening" in this Symposium. As a preliminary experiment we have constructed a prototype apparatus called "Photomicroscanner" which can automatically measure a given defined area and optical density in the classical sense under the microscope exactly.

Devices for obtaining optical density are installed in the circuit of the "Photomicroscanner". Variances of optical density by cell scanning are recorded by an XY recorder. The total sum of optical densities in the area of a cell or a nucleus is adopted as the indicator of optical density and is designated as "total optical density." The area and the total optical density of a cell or a nucleus are calculated automatically and are displayed by the digital display units. In this method, pattern recognition of a cell must be performed by a man.

As for the results of measurements of nuclear area and nuclear total optical density of vaginal smears of a case of uterine cancer stained by toluidine blue alone, there seemed to be a linear relationship between the area of nuclei and the total optical density of nuclei. We were not able to separate cancer cells from normal cells in the single staining method because of lack of information. Sputum specimens stained by Papanicolaou's method were then measured. A number of squamous cancer cells, adenocarcinoma cells, undifferentiated cancer cells, normal squamous epithelial cells, polymorphonuclear leukocytes, lymphocytes and histiocytes were measured automatically. In the specimens stained by nuclear and cytoplasmic staining methods, the following parameters based on geometry and densitometry are available i.e., the cell area, area of a nucleus, nuclear/cytoplasmic ratio, total optical density of cytoplasm, total optical density of a nucleus, average optical density of cytoplasm and the average optical density of a nucleus. Proper threshold values were set up tentatively for each parameter in order to separate cancer cells from other normal cells. However no parameters were found to be sensitive or specific for three cancer cell types. Overlapping distributions of cancer *cells and normal cells ranged from 50 to 90 per* cent. Criteria for diagnosing cancer cells were then detected by combining these seven parameters. When pleural parameters were combined by logical products, a considerable number of combined criteria were found to be more sensitive or specific than single parameter criteria. Selective adoption of these criteria has not been established yet, because the cell populations measured were insufficient in number to decide upon definite combined criteria. From the results obtained to date, however criteria combined with parameters of large cellular size, large nuclear size, and thin cytoplasmic optical density could be rather sensitive those including a large nuclear cytoplasmic ratio could be relatively specific for diagnosing cancer cells.

From the above-mentioned discussion, it would be clear that Papanicolaou-stained preparations could present more *sensitive and specific criteria* to separate cancer cells by automatic processing even though the instrumentation for automated cytodiagnosis should be elaborated. As mentioned in Chapter VI "Approaches to Prescreenistry is rapidly becoming quantitative science. Many of the earlier histochemical methods are proving to be unsuitable for quantitative studies, as many reactions have not been fully investigated. This is true of the phosphatases generally and of the acid phosphatases in particular This enzyme reaction has been studied in great detail and it is possible by investigating its kinetics to quantify the histochemical reaction When applied to the acid phosphatase activity of alveolar macrophages in normal and irritated lungs[2] this technique can be used as an extremely sensitive bio-assay of irritation The cells are scanned by Vickers M85 scanning micro-densitometer (Fig 6) which integrates the density of the final reaction product

A dose-related response to sulphur dioxide and cigarette smoke irritation has been obtained (Fig 7) This technique may be expedited by the use of Zeiss scanning photometer and on-line computer (Fig 8)

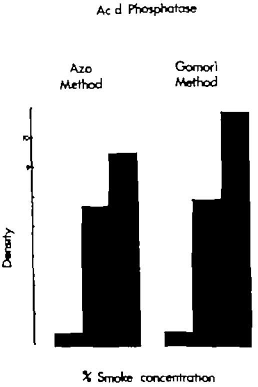

FIG. 7 Acid phosphatase activity demonstrated by two histochemical techniques, in macrophages from control rat lungs and lungs exposed to cigarette smoke.

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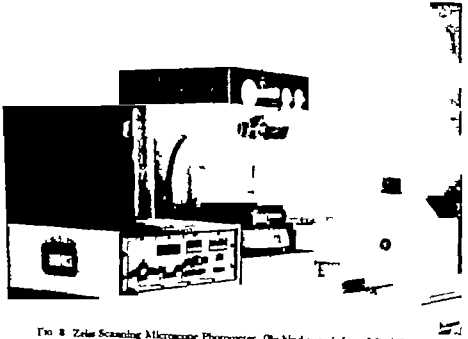

FIG. 8 Zeiss Scanning Microscope Photometer (By kind permission of Carl Zeiss, Degenhardt & Co. Ltd London)

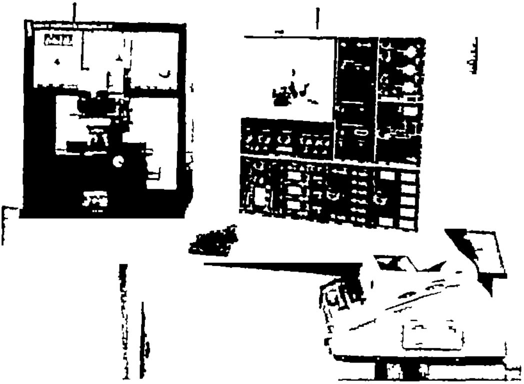

FIG. 5. Image Analyzing Computers—Quantimet 720.

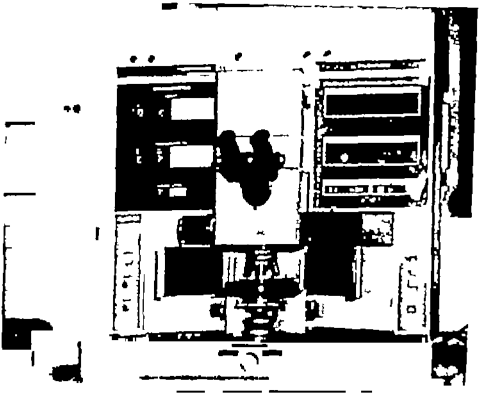

FIG. 6. Vickers M85 Scanning Microdensitometer

by a dark blue final reaction product which can be area sized with the Quantimet B.[7, 8] This technique affords a valuable example of the usefulness of histochemical methods in the quantitative evaluation of toxic changes.

The principle reason that enzyme histochemistry has played a subordinate role in the quantitation of altered cellular function has been that until recently evaluation was subjective and qualitative. This is no longer the case and enzyme histochem-adherence of cells to one another restricts the usefulness of the above methodologies in this area.

Finally with regard to interferometric studies, these are less liable than those employing ultraviolet absorption to error from light scattering etc and combined with specific extraction procedures lend themselves to quantitation of specific chemical substances in cells Thus the amounts of DNA and RNA in cells may be expressed in absolute units, an improvement on the arbitrary units presently necessary with the Feulgen method In addition, an elegant procedure for the study of the kinetics of certain phosphatases is possible with this approach For these reasons it seems pity that the original work[5] demonstrating the feasibility and potential of this technique has not been pursued as vigorously as those employing absorption

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Dr. van Duijn

In principle any property or set of properties of tumor cells can be used for their characterization and therefore can aid in diagnosis. In recent years a great number of cytochemical staining methods[2] have been described, but only a fraction of them have been tested for their discriminatory power in cancer diagnosis, generally in a limited field. In applying these methods, staining intensity has usually been evaluated by visual estimation When the cells one wishes to differentiate show large differences in staining intensity this approach can produce valuable results. It was found, g by Willighagen that the esterase activity of moderately differentiated squamous carcinoma of the lung showed large differences in staining intensity which were found to be correlated with survival time Visual grading of cytochemical tests was shown to produce parameters which, when statistically analyzed with computer program, proved valuable in the classification of leukemias When the enzyme present in cytoplasmic granules is stained cytochemically as is the case of alkaline phosphatase in neutrophilic leukocytes, the visual estimation can be objectivated to some extent by scoring the intensity of the staining of the granules according to prefixed criteria

Such semi-quantitative scoring of the staining method for alkaline phosphatase in neutrophilic leukocytes with the method according to Kaplow has proved its value in diagnostic problems

With this procedure apparently valid data on the enzyme content are obtained, since the Kaplow score was found to correlate well with biochemical alkaline phosphatase determinations in these cells Such favorable conditions for visual estimation, however seem to be limited, since in general the human eye is weak in integrating staining intensity when the chromophores are distributed over variable areas As stressed by Sandritter and Kiefer reliable cytophotometric determination of cell components for diagnostic purposes on larger scale, have thus far been largely confined to the Feulgen reaction for DNA RNA determination with the gallocyanine chromalum method and ultraviolet cytophotometry of nucleic acids has been applied but in more restricted field.

In view of these still limited applications, the full potential of quantitative cytochemistry for cancer diagnosis can by no means be considered as having been put to a definite test. Progress in this field seems to be hampered by at least two factors

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DR. ROSENBERG

The paper by Sandritter and Kiefer clearly points out the value of quantitative DNA and RNA measurements in the study of cells. Measurements of the DNA and RNA content of carefully selected isolated cells may indeed yield information of diagnostic value It should be stressed however that in general one requires isolated individual cells on which to perform these measurements and that such cell preparations are difficult to obtain in a practical diagnostic setting In many cases, such as in the analysis of solid tissues or of cervical aspirations in which cells are clumped in mucoid material, isolated cells cannot be easily obtained. A polyploid cell might well yield values of DNA or RNA content similar to that of two or more attached or overlapping cells. This inability to distinguish single from clumped cells has been one of the greatest difficulties encountered in the use of the "flow photometers" mentioned by Sandritter and Kiefer

The need for better methods for the preparation of cell suspensions is a problem which will probably be dealt with in greater detail elsewhere in this Symposium. It is clearly one of the pressing problems facing the field of automated cytology today

DR STEWARD

In their paper Drs. Sandritter and Kiefer rightly emphasize the prominence and importance of chemical quantitation, and particularly DNA microphotometry

However a consideration of classical quantitation of cell properties should include work relating to the quantitation of morphologic features. This particularly applies to the statistical evaluation of discrete components in a given volume of tissue or cytoplasm i e cell populations, number of mitochondria in a cell, etc. Many methods are available[1 4 4 6 1] but all of those relating to quantitation of the usual microscopic preparation are perforce indirect, and attempt to proceed from a count of random transections to an accurate estimate of the number of whole components in the volume surveyed This poses difficulties. Further most of these methods depend for their accuracy on a random distribution of the components being studied, a situation which is not always realizable, and because of computational difficulties the components are often "idealized" to simple geometric forms Similarly the direct enumeration of cells by a blenderization procedure[8] or by a centrifugation method suffers from a number of theoretic and practical disadvantages Nevertheless, though each method has its own particular limitations, it can when properly applied, be of considerable use

Further although perhaps not yet truly classical are those methods employing computerized systems for the morphometric analysis of cells. These have yet to be fully evaluated, but appear promising Significantly interest in this area has focused on the intact cell and by comparison no work has been done to quantitate necrosis, cell debris, etc features often used as one of the criteria for malignancy

Additionally the work described by the authors deals mainly with cell populations, in which the cells are separated from one another Upon these frequency distributions are derived and statistical analyses made This has much to recommend it, but often, as indicated elsewhere in this symposium, the problem is that of detecting a very few abnormal cells in a large population which is being screened This, plus such factors as the adherence of cells to one another restricts the usefulness of the above methodologies in this area.

Finally with regard to interferometric studies, these are less liable than those employing ultraviolet absorption to error from light scattering etc., and combined with specific extraction procedures lend themselves to quantitation of specific chemical substances in cells. Thus the amounts of DNA and RNA in cells may be expressed in absolute units, an improvement on the arbitrary units presently necessary with the Feulgen method. In addition, an elegant procedure for the study of the kinetics of certain phosphatases is possible with this approach. For these reasons it seems a pity that the original work demonstrating the feasibility and potential of this technique has not been pursued as vigorously as those employing absorption.

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In view of these still limited applications, the full potential of quantitative cytochemistry for cancer diagnosis can by no means be considered as having been put to a definite test. Progress in this field seems to be hampered by at least two factors.

Until recently cytophotometry of large numbers of cells was still too laborious and specialized to find large scale application in a great number of laboratories. Such a large scale application and correlation of the data with clinical observations is a *conditio sine qua non* for development of the field

A second factor inhibiting application of other staining methods than the few applied so far might be the lack of confidence in the specificity and stoichiometry and consequently in the reproducibility of many propagated methods. In this respect the situation is clearly different from the field of clinical chemistry where, over the years, a number of analytical techniques of proven value have evolved.

It now seems possible to strengthen the analytical foundations of cytochemical methods by accurately studying the several quantitative aspects of staining reactions on model systems of known chemical composition and increasing complexity Such studies have been initiated using cellulose films with covalently linked amino acids or nucleotides attached to them, or polyacrylamide films in which macromolecules such as nucleic acids and enzymes have been trapped in the polymer structure [6] This type of approach makes it possible to define the conditions under which a staining method is reliable in the analytical sense since the model systems can be studied biochemically on aliquot parts of films that are cytochemically stained Even calibration of the staining intensity in biochemical units is possible [7]

Though at present only applied to a few staining methods, the model approach opens up many possiblities to study the quantitative chemistry by staining methods already described Only through the application of a large number of fully tested staining methods on a great variety of clinically well documented material a firm *empirical basis for an evaluation of the diagnostic* potentialities of quantitative staining cytochemistry can be laid

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CLOSING REMARKS

DRS SANDRITTER AND KIEFER

It is interesting to note what happens if you are invited to write an article about this problem. There have been interesting questions from Dr Bartels and others, but I think it was clear enough in our article that we want to stand apart and try to find out through *experiments* in which way we can solve the problems of automatic cytodiagnosis

1 We have now just finished a flow cytophotometric machine with Feulgen fluorescent stained cells.
2. We do not know how many cells in a cervical smear under our suspension conditions exceed the borderline DNA content between normal and cancerous cells This is in our program
3 How many cells are let us assume, over the triploid value in normal patients, cancerous, or precancerous lesions is unknown, and this is the problem which has to be solved by experiments, which are now being undertaken

All this is not a problem of accuracy and economy If the machine shows no more than 50 per cent of high range DNA values, then it is of little use for our problem But in this case we have a good quick machine for determining DNA of tumor cell populations for research

To *Dr Bartels question on gallocyanin The* machine works with Feulgen fluorescence

To Dr Rosenberg We have had a lot of trouble with clumped cells, but we think that we have *found a good method to avoid clumping*

The remarks of Dr van Duijn are interesting and these ideas should be taken into account for future work.

In summary I should like to say that our method on automated cytology is an endeavor to strike out in fresh ground.

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Aspiration Biopsy of Tumors of the Neck
II Cytologic Findings in 13 Cases of Carotid Body Tumor

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Department of Otolaryngology, Radiumhemmet, and Department of Clinical Cytology, Institute of Tumorpathology, Karolinska Sjukhuset, Stockholm, Sweden

Carcinoma deposits in palpable lymph nodes can readily be diagnosed by cytologic study of material aspirated from the nodes. The report of a lymph node metastasis is fully reliable when the aspirate contains lymphatic elements in addition to carcinoma cells. When lymph cells are absent, however, the cytologic diagnosis depends upon the clinical judgement that the palpable structure was, in fact, a lymph node. This decision may be difficult and can have serious diagnostic implications in the region of the neck, where the branchial cysts and carotid body tumors may clinically simulate lymph node lesions and at aspiration biopsy yield cells which may be misinterpreted as carcinoma cells.

Branchial cyst aspirates contain squamous epithelial cells, possibly mixed with granulocytes and/or lymphocytes. The difficulties which can arise in the cytologic differentiation of branchial cysts from metastases of highly differentiated squamous cell carcinoma were discussed in an earlier paper.

Aspirates from some carotid body tumors contain cells in microadenomatous structures and can be mistaken for metastatic adenocarcinoma. In order to investigate the feasibility of differential diagnosis between carotid body tumor and metastatic adenocarcinoma on cytologic smears, we have reviewed the biopsy aspirates prepared from 13 histologically verified carotid body tumors. The results are presented in this paper. The feasibility of preoperative diagnosis of carotid body tumor by aspiration biopsy is discussed, together with the possible risk entailed by aspiration biopsy in this tumor.

GENERAL CONSIDERATIONS

Carotid body tumor develops from a chemoreceptor organ (glomus caroticum, carotid body) which most often is located on the median aspect of the internal carotid artery close to the bifurcation and is embedded in perivascular connective and fatty tissue. It is a rare neoplasm. The largest series in the available literature are the 18 cases presented by Peterson and Meeker,[14] 19 by Harrington, Clagett and Dockerty, 17 by LeCompte,[23] 22 by Lahey and Warren,[19] 29 by Conley, 37 by Farr[11] and 47 by Pettet, Woolner and Judd.[28]

The tumor may appear at any age, but is seen mainly in middle-aged persons of both sexes. It may be bilateral.

Carotid body tumors mimic to a variable extent the histologic structure of the carotid body. LeCompte[23] classified them histologically into three groups: 1) the usual type reproducing the normal structure (cfr Fig. 1a), 2) the adenoma-like type with cells resembling epithelium (cfr Fig. 2), and 3) the angioma-like type with spindle or crescent-shaped cells simulating endothelial cells (cf. Fig. 3). Though the vascularity may vary in different parts of the tumor, there is as a rule a rich network of blood vessels surrounding islets of tumor cells.

Tumors of the carotid body usually are clinically benign. Malignant manifestations such as metastases in regional lymph nodes and in distant organs have, however, been described.

PRESENT SERIES

Thirteen patients underwent aspiration biopsy of carotid body tumor at Karolinska Sjukhuset during the years 1960 through 1968. Nine were women and four men. Their average age was 47.7 years (range 19 to 79 years). Eleven of the tumors were subsequently operated on at this hospital and two at other hospitals. In one case the tumor was malignant with regional lymph node metastases. A brief report of this case has been published elsewhere. Distant metastases have not so far been found in any case.

RESULTS

The initial cytologic reports in the 13 cases of histologically verified carotid body tumor are presented in Table I, where the reports from the biopsies performed during the last two years of the study period are shown separately.

In one case early in the study the aspirate contained only blood. Metastasis of thyroid carcinoma was suspected in two cases, neurofibrosarcoma in two cases and neurofibroma in one case, all from

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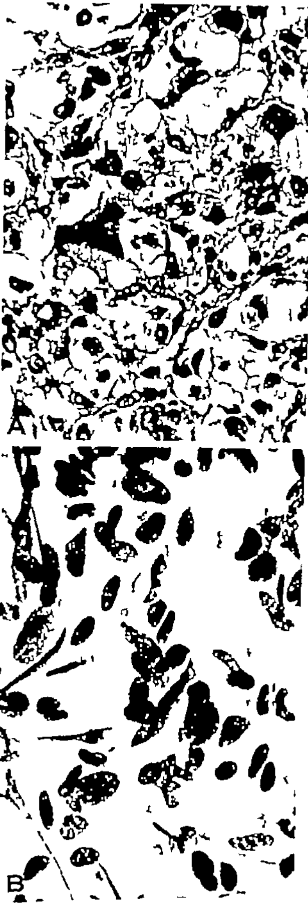

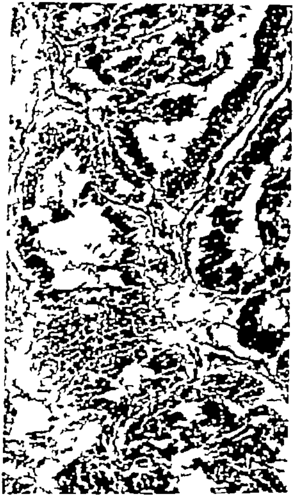

FIG. 2 Carotid body tumor adenoma-like" type The general pattern and cellular morphology strikingly resemble epithelium. Tissue section Van Gieson stain ×160.

the first years of the study Carotid body tumor was cytologically indicated in seven cases, four of them from the last two years

The aspirates, which were obtained with a 22 gauge needle, consisted of a drop of blood with in most cases macroscopically detectable tissue fragments on the smears *Microscopic review showed* fairly numerous tumor cells in eight cases and scanty cells in four cases. The tumor cells formed variously-sized clusters They had abundant cytoplasm and indistinct cellular borders (Figs. 1b and 4b) In some cases the cytoplasm contained variable amounts of granules which took on a reddish tinge with May-Grünwald-Giemsa stain (Fig 4e and f)

FIG 1 (a b) Carotid body tumor usual type"

) Tissue section from an area with pronounced cellular polymorphism. Van Gieson stain ×400. *b*) Aspiration biopsy smear from the same tumor showing syncytium with pronounced nuclear polymorphism. Papanicolaou stain ×640.

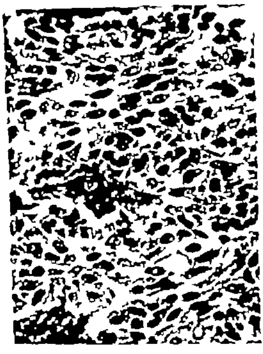

FIG. 3 Carotid body tumor [illegible] type These section from an area showing oval or spindle-shaped cells Van Gieson stain ×400

TABLE I *Initial Cytology Reports in 13 Histologically Verified Cases of Carotid Body Tumor*

Cytologic reports	[illegible]	[illegible]
Only blood in smear	1	
Suspected metastasis of thyroid carcinoma	2	
Suspected neurofibrosarcoma	2	
Suspected neurofibroma	1	
Carotid body tumor	3	4
Total	9	4

Pronounced anisonucleosis was present in all 12 ases and in five of them huge nuclei were present (Fig 4c) The nuclei were usually round or ovoid, but could be of spindle or crescent shape (Fig 4f) The presence of spindle-shaped nuclei could explain the cytologic suspicion of neurofibromatous tumor in three cases

1 of the 13 cases of carotid body tumor areas ere found in the smears where the cells were arranged in acinous or follicular structures (Fig 4d) These structures suggested the—erroneous—suspicion of metastasis of thyroid carcinoma in tw cases

DISCUSSION

The clinical appearance of carotid body tumor is that of a smooth, or rarely nodular swelling of varying size lying beneath the anterior part of the sternomastoid muscle and below the mandibular angle. [illegible] In many cases it may be observed as a bulging of the lateral pharyngeal wall. [illegible] Because of the tumor's intimate relation to the carotid arteries, the arterial pulsations generally are transmitted through it and may be felt on palpating the swelling[illegible] The tumor usually is mobile in horizontal but not in vertical direction [illegible] It grows slowly and the patient may have observed hump in the neck for years before seeking medical advice, other symptoms being absent. [illegible] Later on, pressure from the tumor or invasion of adjacent structures may give rise to hoarseness, dyspnoea, cough, headache or Horner's syndrome [illegible] Many writers have stated in the past that correct preoperative diagnosis is rarely made [illegible]

Aspiration biopsy has therefore been recommended for the diagnosis of carotid body tumors [illegible] When this tumor is suspected and transmitted arterial pulsations can be felt, aspiration biopsy must be cautiously contemplated The reason is the risk of complications due to the rich vascularity of the tumor and its close proximity to the large carotid arteries [illegible] One case of the present series illustrates this risk.

In 33-year-old man, aspiration biopsy of a suspected carotid body tumor of the right side yielded only blood. Six days later he became hemiplegic Angiography showed complete obstruction of the common carotid artery At an emergency operation a large carotid body tumor was extirpated and resection of the carotid arteries was performed Thrombosis of the internal carotid artery was present The excised tissue showed carotid body tumor and fresh bleeding between the intimal layer of the internal carotid artery and invading tumor tissue The patient died three days later Necropsy revealed 12 mm long thrombus in the right median cerebral artery presumably due to embolism In the right hemisphere there was an orange-sized area of malacia

Though it is not certain that the embolus was caused by the aspiration biopsy this case underlines the risks in needling of large carotid body tumors

We therefore suggest that if a carotid body tumor is clinically suspected, aspiration biopsy should not be performed Instead carotid artery angiography is recommended, which reveals the typical picture of a richly vascularized tumor widening the space between the internal and the external carotid artery [illegible] Engzell re-

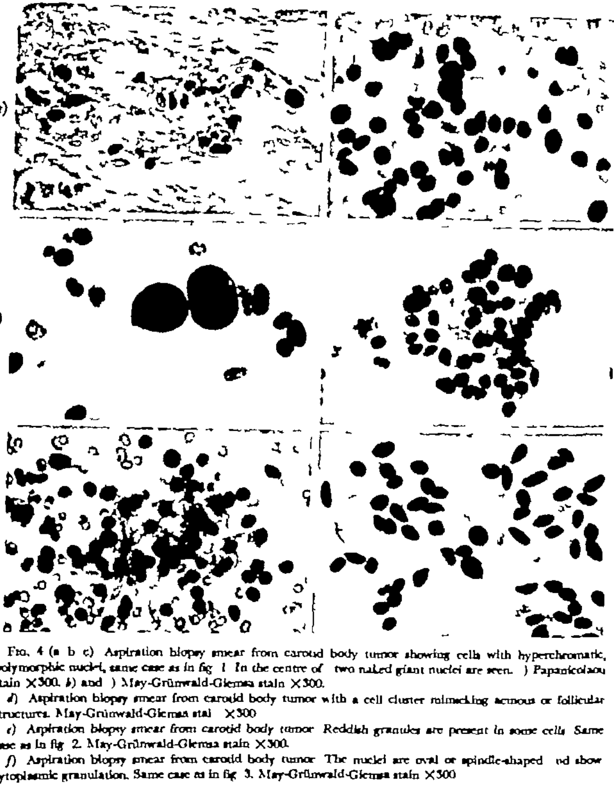

FIG. 4 (a b c) Aspiration biopsy smear from carotid body tumor showing cells with hyperchromatic, polymorphic nuclei, same case as in fig 1 In the centre of two naked giant nuclei are seen.) Papanicolaou stain ×300. *b*) and) May-Grünwald-Giemsa stain ×300.

d) Aspiration biopsy smear from carotid body tumor with a cell cluster mimicking acinous or follicular structures. May-Grünwald-Giemsa stain ×300

e) *Aspiration biopsy smear from carotid body tumor Reddish granules are present in some cells Same case as in fig* 2. May-Grünwald-Giemsa stain ×300.

f) Aspiration biopsy smear from carotid body tumor The nuclei are oval or spindle-shaped nd show cytoplasmic granulation. Same case as in fig 3. May-Grünwald-Giemsa stain ×300

ported a definite preoperative diagnosis in this way in all nine cases of a series of carotid body tumors

When carotid body tumor is not suggested by the clinical examination, or if angiography does not indicate this tumor aspiration biopsy will be done. The differential diagnosis then involves numerous other lesions such as branchial cyst, lymphadenitis, neurofibroma, neurofibrosarcoma malignant lymphoma and lymph node metastases from carcinoma or sarcoma above or below the clavicle.[11] 1 1 14 14 4 6 In our experience, cytologic examination of aspirate from clinically undefined swelling in the carotid region readily permit differentiation between such lesions as branchial cysts, lymphadenitis, malignant lymphomas and metastases of squamous-cell carcinoma and carotid body tumors.

Misinterpretation of carotid body tumor as primary neurofibroma or neurofibrosarcoma must be considered. In one of our cases, the presumed cytologic diagnosis was neurofibroma and in two it was neurofibrosarcoma. This was apparently attributable to the presence of ovoid and spindle-shaped nuclei with pronounced polymorphism. When this cytologic picture is seen in aspirate from a tumor of the carotid region, the cytologist should request that angiography be done to clarify the diagnosis.

Differentiation between adenocarcinoma and carotid body tumor seems to call for some comment, since in two of our 13 cases of carotid body tumor the smears from aspiration biopsy were initially thought to show metastases from thyroid adenocarcinoma. Cell clusters mimicking acinous or microfollicular structures were responsible for this misjudgment. Morphologic comparison of such slides with aspirate from true metastatic follicular carcinoma of the thyroid, however, clearly reveals the difference. In the latter case the cells are more densely packed and the nuclei are of more uniform size, polymorphism as a rule being absent. If the cytologist is uncertain, careful examination of the thyroid, including scintiscan, should be performed. Any palpable swelling in the thyroid or cold nodule can be punctured and smears from the aspirate can be compared with those from the swelling in the carotid region. Confirmation of a cytologic diagnosis of carotid body tumor by carotid angiography will rule out the possibility of metastatic thyroid carcinoma.

SUMMARY

Fine-needle (22-gauge) aspiration biopsy smears from 13 cases of histologically verified carotid body tumor were reviewed. The aspirate contained tumor cells in 12 cases. The morphologic details by which these tumors can be cytologically diagnosed from smears of aspirate are described. The risk involved in needling tumors intimately related to the carotid arteries is pointed out.

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ACTA CYTOLOGICA
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Cytologic Diagnosis of Renal Cell Carcinoma with the Aid of Fat Stain

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The cytologic examination of urine sediment is frequent component of urologic investigation. Although smears prepared from the sediment of alcohol fixed urine and stained by the Papanicolaou technique are employed with good results for the diagnosis of ureteral and bladder tumors, the same technique has been found less than satisfactory for the diagnosis of renal cell carcinoma.

Renal cell carcinomas are the most common malignant neoplasms of the kidney. They are considered to be slowly growing and are often clinically silent. Renal cell carcinomas producing symptoms frequently reach the diameter of several centimeters; only 25 per cent of the patients live five years or longer after diagnosis. At present, the routine cytologic procedure has little to offer early diagnosis of renal cell carcinoma.

The purpose of this report is to summarize simple method of processing and staining the urinary sediment. The method appears promising with regard to diagnosis of renal cell carcinoma.

TECHNIQUES

A fresh sample of urine, at least 10 ml, is collected in clean container and delivered to the laboratory within an hour without adding any fixative. The unfixed urine is centrifuged at 2000 rpm for 25 minutes and at the end air-dried smears are prepared from the sediment. Smears were kept at room temperature for several hours before staining with oil red-O.

Staining procedure (modified oil red-O stain)

1. Absolute propylene glycol, two dips
2. 0.5 oil red-O solution for two hours (0.5 gm oil red-O diluted with 100.0 ml 100 propylene glycol)
3. Differentiate in 85 propylene glycol solution for 30 seconds
4. Rinse in distilled water, one change
5. Stain in Harris' hematoxylin solution for 10 seconds
6. Rinse in distilled water, changes
7. Dip in weak ammonia water, two dips
8. Wash in water, two changes
9. Mount with glycerin jelly

With cooperation of the Urology Service of the Department of Surgery, this technique has been employed in addition to routine urine cytology in 20 patients who were admitted to Memorial Hospital with the clinical diagnosis of renal neoplasm. In addition, urine samples were collected and processed similarly to the above from 22 patients without apparent evidence of renal neoplasm. In this group we included patients having pyelonephritis, cystitis, hydronephrosis, carcinoma of bladder, carcinoma of prostate, and diabetes.

RESULTS

Oil red-O positive cytoplasmic granules were observed in 14 of the 20 clinically suspicious patients. All 14 proved to have renal cell carcinoma. The tumor was also present in three additional patients with negative sediment (Table I). Of the remaining three patients, two had epidermoid carcinoma of the renal pelvis and one had renal cyst.

Routine cytologic smears prepared from the sediment of alcohol fixed urine and stained by the Papanicolaou technique were reported negative for malignant cells in all but one of the 17 patients with histologically proved renal cell carcinoma.

None of the smears of the 22 control patients showed positive stain with oil red-O.

The oil red-O positive cells were in general scanty on the smears. These cells were almost always single. Clusters of cells were rarely seen. The oil red-O positive granules were coarse, bright red, distinctly intracytoplasmic and did not polarize (Fig. 1 A, B, C). The nuclei of the positive cells stained pale blue and the nuclear membrane was well preserved. A prominent nucleolus could be easily seen in most of the cells (Fig. 1 B, C and D). The cytoplasm of the fat-positive cells appeared finely vacuolated and colorless except for the sharply demarcated bright red diffusely dispersed oil red-O stained granules.

In a further attempt to identify the cells containing oil red-O granules, touch smears and frozen sections of the subsequently removed renal cell carcinomas were stained with oil red-O. The oil red-O positive cancer cells in these preparation were strikingly similar to the cells with fat granules found in the urinary sediment (Fig. 1D).

TABLE I. *Comparison of Cytologic Findings in 17 Histologically Proved Cases of Renal Cell Carcinoma*

Sediment of unfixed urine stained with oil red-O		Sediment of alcohol fixed urine stained with Pap stain	
Positive	Negative	Positive	Negative
14	3	1	16

All three patients had small cortical renal cell carcinoma without invasion of renal pelvis.

DISCUSSION

The small series of cases presented suggests that oil red-O positive cells are observed only in the sediment of unfixed urine of patients with renal cell carcinoma of the kidney. Such cells have not been identified in the smears of control patients including patients with epidermoid carcinoma of the renal pelvis. The oil red-O positive cells are probably cancer cells which are difficult to identify in smears prepared from sediments of alcohol fixed urine stained by the Papanicolaou technique[1,4,6].

It is well known that in patients with nephrotic syndrome anisotropic extracellular lipids and so-

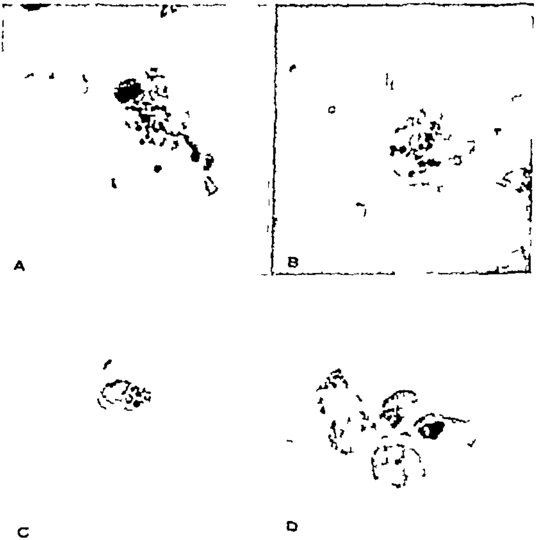

FIG. 1. A, B, C. Coarse intracytoplasmic oil red-O positive (black) granules in bizarre large cells in smears prepared from the sediment of unfixed urine of patient with renal cell carcinoma. D. Touch smear of renal cell carcinoma. Note the strikingly similar cells and intracytoplasmic oil red-O positive granules. (Oil red-O ×560).

called "oval fat bodies" are commonly present in the urine sediment.

Of interest, however, is that the oil red-O positive granules found in renal cancer patients were always intracytoplasmic, isotropic under polarized light; none of the patients had a nephrotic syndrome.

In view of our limited experience and possible existence of oil red-O positive cells in urinary sediment in other disease than renal cell carcinoma, further clinical and laboratory studies are indicated before the presence of oil red-O positive cells in the sediment of urine can be accepted as specific for renal cell carcinoma.

SUMMARY

Oil red-O stained smears prepared from sediment of unfixed urine proved to be superior to present cytologic techniques in identification of exfoliated cells of renal cell carcinoma, in a series of 20 clinically suspicious patients. Attention is called to diagnostic and differential diagnostic implications.

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New York State Computerized Proficiency Testing Program in Exfoliative Cytology Development

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The New York State Laboratory Services Act of 1964 mandated that clinical laboratories obtain a valid permit and maintain minimum Department of Health standards in performance. This included participation in a State-operated proficiency testing program covering a wide range of clinical laboratory disciplines.

In 1968 the Department embarked on the first computerized program of proficiency testing in diagnostic cytology. The purposes were to evaluate the performance of cytotechnologists, to determine any relationship of performance to a variety of laboratory factors, and to relate resultant data to the educational background of cytotechnologists, thus offering the possibility for the establishment of standards applicable to laboratories performing diagnostic exfoliative cytology.

Test specimens consisting of three cervical vaginal and two sputum smears were carried by our clinical laboratory investigators to each laboratory holding a permit in exfoliative cytology for on-site testing of cytotechnologists. Whereas an ideal testing program should include smears from all body fluids, initial limitation to most frequently examined material was considered desirable. With the help of the Office of Electronic Data Processing of the New York State Department of Health, a data processing program was formulated before testing.

It was hoped that such a program would allow for accumulation of data for both baseline information on performance and education of cytotechnologists, and the tabulation of characteristics of cytology laboratories. Figure 1 illustrates the flow chart of the total cytology proficiency testing program.

Selection of smears for proficiency testing was made in our laboratory where at least two pathologists, and sometimes three, examined the smears. This selection was conditional on complete agreement of interpretation. Three additional laboratories, namely, Memorial Hospital for Cancer and Allied Diseases in New York City, the Albany Medical College of Union University in Albany and the Crouse-Irving Memorial Hospital in Syracuse, were chosen as reference laboratories. Each of these laboratories examined specimens in excess of 15,000 annually. Agreement on the interpretation of the smears by all three reference laboratories was a prerequisite as was confirmation by biopsy of all except negative specimens.

Rating of test specimens by each cytotechnologist in a laboratory was based on 20 points per smear and total correct identification of all smears equalled 100 points. Table I illustrates the scoring which might be obtained in the interpretation of all five smears under a variety of circumstances. A passing grade of 80 points was established thus allowing one "20" point error or two "10" point errors in the interpretation of the five smears. The cytotechnologist test form (Fig. 2) was accompanied by an instruction sheet. This instruction sheet was sufficiently detailed to eliminate misunderstanding of the final reporting, and the laboratory investigator was also present to explain any possible ambiguity. Identification and cross reference were insured by use of the laboratory number in the upper right hand corner of the test form as well as by the cytotechnologist's social security number. Figure 3 is the code sheet used to enter the results of the test sheet for data processing. Similar code sheets were used to enter the results from other forms.

A report of the cytotechnologist's test was sent to the director of the cytology laboratory. All errors were tabulated with the State's answers and comments were added to serve as guides for the cytotechnologist and the director of the laboratory. Failure to achieve 80 points made retesting mandatory. If the second testing results were still unsatisfactory, a third test was required.

A cytology personnel questionnaire was devel-

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Presented at the IV Pan American Cancer Cytology Congress, May 30–June 1, 1970, at Ocho Rios, Jamaica, W.I.

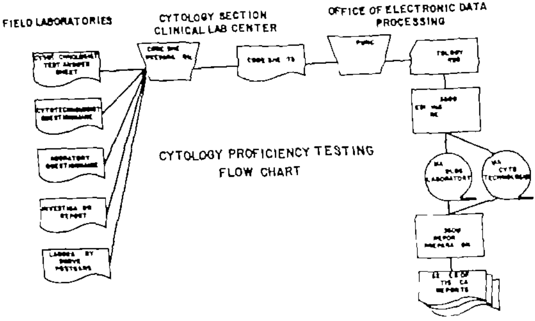

FIELD LABORATORIES
CYTOLOGY SECTION CLINICAL LAB CENTER
OFFICE OF ELECTRONIC DATA PROCESSING
CYTOLOGY PROFICIENCY TESTING FLOW CHART

Fig. 1

Table I *Rating of Smears*

Correct interpretation corresponding to key answer	20 points
Correct answer is positive, biopsy is positive and cytotechnologist checks suspicious	10 points
Correct answer is suspicious, biopsy is positive and cytotechnologist checks positive or suspicious	20 points
Correct answer is suspicious, biopsy is non-confirmatory and cytotechnologist checks suspicious	20 points
Correct answer is negative and cytotechnologist checks positive or suspicious	0 points
Correct answer is suspicious, biopsy is non-confirmatory and cytotechnologist checks positive or negative	0 points

5 smears × 20 points each 100 points
Acceptable performance 80 points

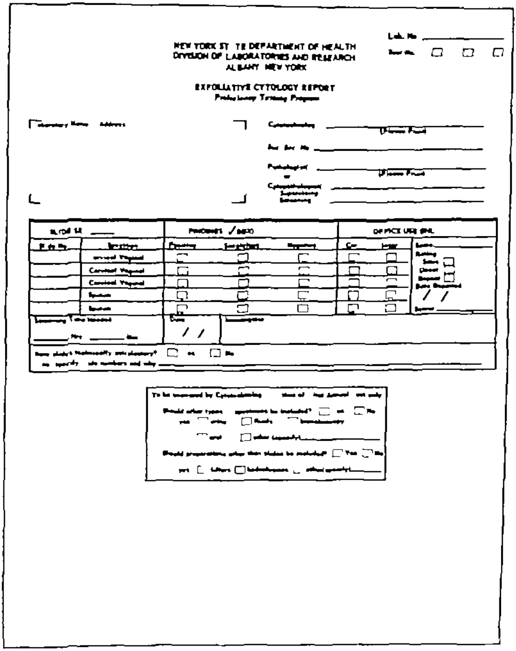
Table II *Distribution of cytotechnologists in permit laboratories*

January 1, 1969 to June 30, 1969	
Number of laboratories with permit in cytology	182
Number of laboratories visited	130
Number of laboratories with cytotechnologists	62
Number of cytotechnologists	111
Number of laboratories without cytotechnologists	68

oped (Fig. 4) to determine the possible relationship of past and present education, training, and experience on the cytotechnologist's performance. Since scope of work in the laboratory, methodology, laboratory procedures (such as Papanicolaou staining and acridine orange fluorescence techniques) in addition to supervision by pathologists, may also reflect on performance, a form was designed to obtain this information as well as type and degree of supervision by the pathologist, availability of follow-up data, and amount of work in each laboratory (Fig. 5).

The clinical laboratory *proficiency testing program* in other disciplines requires our laboratory investigators to transcribe a fairly detailed narrative report of their observations of physical facilities, work conditions, type and quality of equipment, availability of teaching material, and record keeping in the laboratories. Therefore, a similar report was requested about laboratories engaged in diagnostic cytology for evaluation of general standards (Fig. 6). Additional information was included in the general narrative report filed after completion of testing and inspection of other laboratory categories.

As an adjunct to the proficiency testing program, the teaching Kodachrome-of-the-Month



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NEW YORK STATE DEPARTMENT OF HEALTH
DIVISION OF LABORATORIES AND RESEARCH
ALBANY NEW YORK

EXFOLIATIVE CYTOLOGY REPORT

(Please Print)

(Please Print)

	Cervical Vaginal	☐	☐	☐	☐	☐	
	Cervical Vaginal	☐	☐	☐	☐	☐	
	Cervical Vaginal	☐	☐	☐	☐	☐	
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FIG.

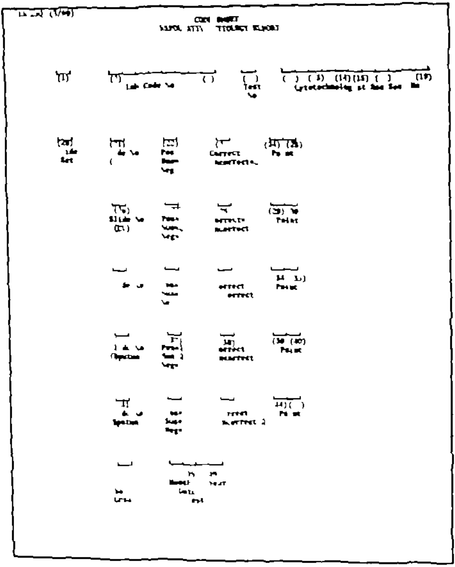
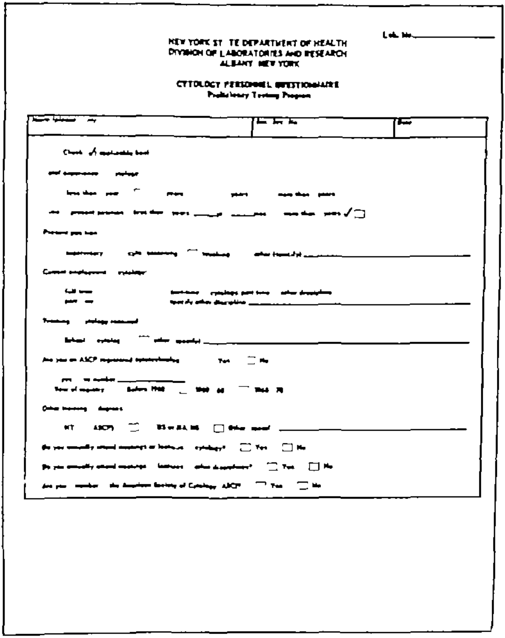


Fig 3



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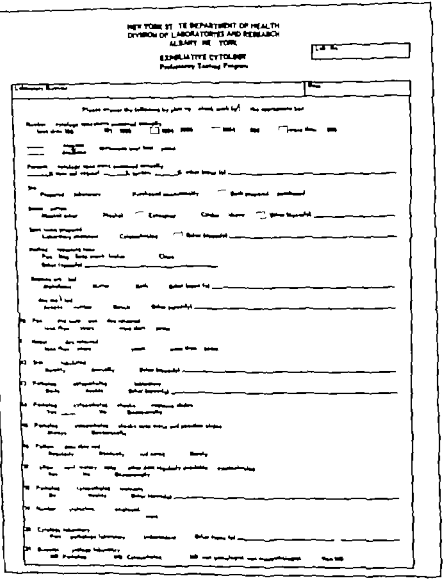
CYTOLOGY PERSONNEL QUESTIONNAIRE
Proficiency Testing Program

FIG. 4



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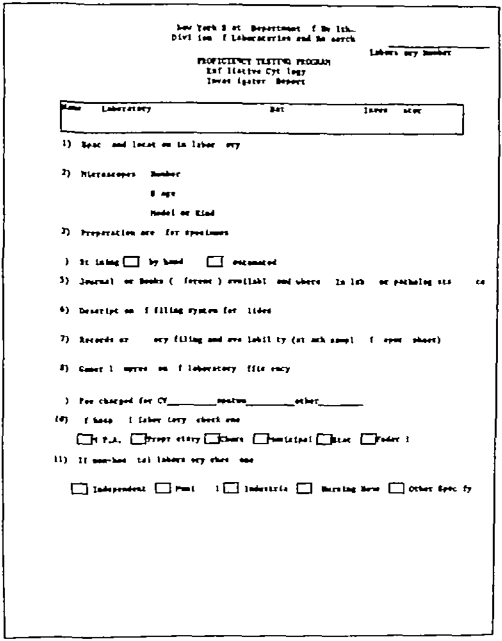
EXFOLIATIVE CYTOLOGY
Proficiency Testing Program

FIG. 5



New York State Department of Health
Division of Laboratories and Research

Laboratory Number

PROFICIENCY TESTING PROGRAM
Exfoliative Cytology
Investigator Report

Name Laboratory Date Investigator

1) Space and location in laboratory

2) Microscopes Number

Age

Model or Kind

3) Preparation area for specimens

4) Staining ☐ by hand ☐ automated

5) Journals or Books (reference) available and where In lab or pathologists [illegible]

6) Description of filing system for slides

7) Records or [illegible] filing and availability (attach sample of report sheet)

8) General impression of laboratory efficiency

9) Fee charged for CY_________ sputum_________ other_________

10) If hospital laboratory check one

☐ N.P.A. ☐ Proprietary ☐ Church ☐ Municipal ☐ State ☐ Federal

11) If non-hospital laboratory check one

☐ Independent ☐ Municipal ☐ Industrial ☐ Nursing Home ☐ Other Specify

FIG. 6

TABLE III *Examination Results Proficiency Testing in Exfoliative Cytology January 1969 to June 1969*

Pass	Number Cytotechnologists	Score	Fail	Number Cytotechnologists	Score
No errors	40	100%	3 10-point errors	1	70%
1 10-point error	44	90%	4 10-point errors	9	60%
2 10-point errors		80%	5 10-point errors	1	50%
			6 10-point errors		40%
			7 10-point errors	1	30%
Total	86			[illegible]5	

series was initiated and this is now in its second year. Interesting and informative cases were distributed to all laboratories with permit in exfoliative cytology. These cases, consisting of one or more photographed fields from smears, were accompanied by histories, discussions and references.

RESULTS

There were 182 laboratories with permits in cytology (Table II). During the initial six-month period of the program 130 laboratories were inspected and 111 cytotechnologists were tested. Sixty-eight laboratories visited employed no cytotechnologists (all screening being done by the pathologist) and 62 employed one or more cytotechnologists. Eighty-six cytotechnologists achieved satisfactory grade, 40 received a perfect score, and 46 showed varying combinations of errors. Of the 111 cytotechnologists participating in this initial testing, 25 failed to attain satisfactory grade (Table III).

SUMMARY

A State-operated computerized proficiency testing program in cytology was initiated by the Division of Laboratories and Research of the New York State Department of Health in 1968. The program was designed to permit accumulation of a wide range of data pertaining to a variety of conditions such as private laboratories, hospital laboratories, laboratory direction by pathologists or nonpathologist physicians, as well as education, training and experience of cytotechnologists. Test specimens were reviewed and selected by two or three department pathologists and three reference laboratories. These slides were carried by laboratory investigators to participating laboratories for screening by cytotechnologists. One hundred and eleven cytotechnologists participated in this first testing program from January 1 1969 through June 30 1969. Eighty-six cytotechnologists passed this initial test and 25 failed. Studies are now in progress to determine possible reasons for these results and to test the validity of the system.

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The Chromosomal Aberration of Double-Minutes in a Human Embryonic Rhabdomyosarcoma

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During the past year we have experimented with fine needle aspiration as a means of obtaining material for chromosomal studies of human solid tumors. The needle used in the technique had an outer diameter of 0.6 mm and was attached to a disposable plastic syringe fitted to a special aspiration instrument[1] The major part of the aspirate was taken for routine cytodiagnosis, and the remainder was processed for chromosomal studies. In spite of the small amount of material available satisfactory results were obtained in several cases. One of these was a metastatic embryonic rhabdomyosarcoma, of particular interest because of the presence of the chromosomal aberration of double-minutes (dms) This rare anomaly[2, 3, 5] is characterized by the occurrence of varying numbers, of usually extremely small paired chromatin bodies in addition to the ordinary chromosomes. At present the origin of these dms and their propagation in the tumor cell population are controversial questions The possible existence of a centromere, in at least some dms, is a problem related to the last question[2, 4, 5]

MATERIAL AND METHODS

Following preoperative irradiation an embryonic rhabdomyosarcoma (Fig 1) in the right infraclavicular region was completely removed from a 16-year-old boy in June 1969 At operation metastases were found in the right axillary lymph nodes This region was therefore included in the postoperative field of irradiation Two months later a fine needle biopsy showed metastases in the left axillary lymph nodes (Fig 2) Subsequently this region and the upper part of the chest were irradiated The patient died in January 1970 with extensive pulmonary metastases Cytostatics were not used. The material for chromosomal study was taken from the needle biopsy of the metastases in the left axillary lymph nodes. The aspirate was suspended in five ml of Parker's medium 199 (COLCEMID, CIBA) added to a final concentration of 1.0 μg per ml, and incubated for one hour at 37 C The method used for the chromosome preparation was described earlier[6] All metaphases were counted two or more times Karyotype analyses were made by photography

RESULTS

Numerical findings

Chromosomes were studied in 70 cells. The counts of normally sized chromosomes are shown in Table I last row About one-third of the metaphases had the stemline (S) number $S = 60$, and another third had the numbers of 59 and 61 The other variant cells were found mainly in the hypomodal region. A wide range of chromosome numbers in the hypotriploid and hyperdiploid-diploid regions were represented. This finding could have been explained by counts of broken cells. Karyotype analyses, however indicated that it was a true hypomodal variation. Doubling-products of S-cells or related variant cells were not found.

Dms occurred in all of the 70 cells (Table I upper rows) The number of dms per cell varied from between 3 to 4 to about 60 but only a minor fraction, less than 15 per cent of the cells, had numbers above 30. Below 30 there were two flat modes, one at 5 to 10 dms and the other at 15 to 20 dms. In this context it must be mentioned that some of the dms were extremely small and that in crowded metaphases it was impossible to exclude that a few might have been obscured by ordinary chromosomes. These discrepancies have been accounted for in Table I where the distribution of dms on each chromosome number class have been restricted to a few ranges These figures show that no particular range of numbers of dms was related to any special chromosome number The similarity of the distribution of dms in different chromosome number classes was a finding supporting the clonal origin of the tumor cells

The presence of modes for the dms demonstrate *that they are at least to some extent, influenced* by the mitotic mechanism A previous study however showed a frequent lagging of the dms during anaphase and during telophase the formation of often very small micronuclei by the lagging dms. In the present tumor 400 cells in interphase were studied with regard to the occurrence of micronuclei. They were present in about eight per cent of the cells. More than four micronuclei were seen in a few cells but in most cases there were only one or two. The size of the micronuclei was variable but most of them were as small as those seen in the murine Rous sarcomas[1] and in one human neuroblastoma

Structural findings

Complete karyotype analyses were made in 17 cells. Thirteen of these had the chromosome numbers of 59, 60 and 61. The karyotype of a stemline cell is shown in Figure 4 a: there was a gain of one chromosome in groups E and G, a gain of two chromosomes in groups A (two extra A2), B, C and D, and four extra chromosomes in group F. In relation to the S-karyotype all 61-chromosome cells analyzed showed a further extra chromosome in group C (Fig. 4 b). The 59 chromosome cells studied differed as regards their D and E groups in addition to group C (Fig. 4). The other four tumor cells analyzed were closely allied to those discussed above except for the one with 46 chromosomes. In this pseudodiploid cell, there was a gain of one C chromosome and a loss of one G chromosome. The karyotype analyses suggested multipolarity leading to the development of the hypotriploid tumor cell population.

No markers other than dms were seen. The dms appeared as pairs of small dots or rods. A few of them approached the G chromosomes in size (Figs. 3 and 4 a–c). The majority of the dms, however, had a size equal to or somewhat smaller than the arms of the F chromosomes, and a considerable number of them were close to the border of visibility. The smaller dms especially often showed fuzzy borders which seemed to be due to increased stickiness. The members of each pair of dms were often more widely separated than the chromatids of ordinary chromosomes (Figs. 3 and 4 a–c). This feature was very frequent in cells in middle and late metaphase. Even in early metaphase there were usually some dms with more or less wide separation of their "chromatids". A thread-like connection between the "chromatids" was seen in a few dms in many cells (Fig. 4 a–c, arrows). A clear centromere was not observed. In all the cells studied the dms were even distributed in the metaphase spreads, and clusters of dms[1] were not seen.

Chromosome and chromatid breaks occurred in a frequency of below five per cent. Pulverization centres, reported earlier were not found. The dms always appeared as solid dots or rods and fragmentation was not observed.

DISCUSSION

To date, only eleven human neoplasms with the aberration of dms have been reported (for references see /6/). These cases showed special characteristics as regards the age of the patients and the

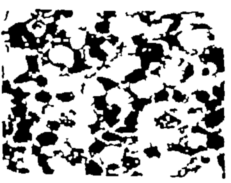

FIG. 1 Biopsy from the infraclavicular embryonic rhabdomyosarcoma before irradiation (H & E stain) ×400

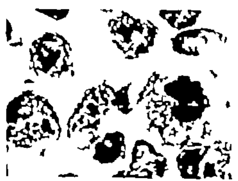

FIG. 2 Fine needle aspirate from the metastases in the left axillary lymph nodes (Eosin ×1200)

TABLE I Number of dms in relation to counts of ordinary chromosomes (last row)

Number of dms	Chromosome numbers															Total cells
	46	49	50	51	53	54	55	56	57	58	59	60	61	62	70	
5–10		—	—	—												
20						—	—	—	—	2	2	4	4	1	—	1
21–30	—		—		2		—	2	1	2						27
31–40					—	2	2	1	—	—	1	5	4	—	—	6
41–50			—			—		—	—	—	1	2	—	2	—	
51–60		—	—	—	—	—	—	—	—	—		2	—	—		
							—	—	—	—	—	—	1	—	—	1
5–60		1		2	2		2	3			8	22	14		1	70

histopathologic types. Thus, seven of the neoplasms occurred in children and nine tumors were neurogenic. The present case reemphasizes the occurrence of dm+positive neoplasms in young patients, usually children. However one of the two nonneurogenic tumors with dms reported previously was a facial rhabdomyosarcoma.[illegible] This sarcoma had a near-triploid stemline, but the stemline karyotype (ordinary chromosomes) was completely different from that of the present case. Nevertheless, it is interesting to note that the anomaly of dms has been seen in two of the six rhabdomyosarcomas, so far subjected to chromosome analysis [illegible] Additional cases will clarify whether this is an incidental finding

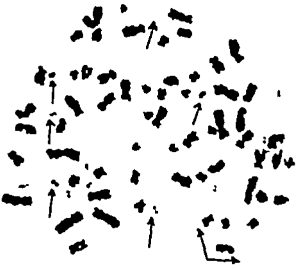

FIG. 3 Metaphase of a variant cell, 2x = 61 with about 30 dms. The arrows point at the 3 biggest dms and at 4 of the smaller ones. The latters often have fuzzy borders and some of them more or less separated "chromatids" ×1 50.

Ever since the detection of the aberration of dms the possibility of an extraneous origin has been considered The present results gave no support to this idea.

In all the cases reported the morphology of the dms has been similar—pairs of small, solid dots or rods without a clear centromere and with a tendency for the "chromatids" to separate. The differences between the tumors are confined to variations in the average number of dms per cell and the size classes dominating in a particular neoplasm. These differences may reflect observations at different evolutionary stages, and they hardly change the impression that one is dealing with the same phenomenon in all cases. Theories regarding the origin of the dms and their propagation in the tumor cell population were discussed recently[4] The present case adds little to the understanding

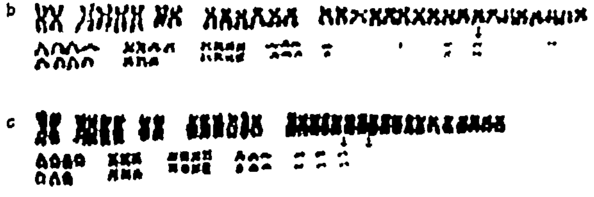

FIG. 4 a Karyotype of a stemline cell S = 60 with 16 dms. b Karyotype of a *hypermodal* variant cell 2n = 61 with 15 dms the arrow point at one pair with a connective thread. c Karyotype of a hypomodal variant cell, 2x = 59 with 8 dms the arrows point at 2 pairs with a connective thread.

of these questions. One point of interest, however, is the possible influence radiotherapy may have on the origin of dms. Thus, the primary tumor was irradiated prior to the present study. Though the material used for the chromosomal analysis was taken from lymph nodes located outside the field of radiation, it can not be excluded that the metastases in the nodes had appeared late and originated from a tumor site inside the former field of irradiation. The low frequency of breakage, the absence of chromosomal interchanges and the absence of markers are findings which make it unlikely that the radiotherapy was responsible for the appearance of dms in the present tumor.

SUMMARY

Material from a fine needle biopsy of a metastatic, embryonic rhabdomyosarcoma in a 16-year old boy was used for chromosomal analysis. The sarcoma had a hypotriploid stemline, and all cells studied showed the aberration of double-minutes (dms). The number of dms per cell varied between 3 to 4 to about 60 but the majority of the cells had less than 30 dms. Irregular mitotic behavior of the dms was indicated by a comparatively high frequency of micronuclei in interphase cells. The origin of the dms was obscure, and the appearance of dms could not be convincingly related to the therapy given.

ACKNOWLEDGMENT

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Malignancy Associated Changes in Buccal Smears*

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Recent studies have broadened the concept of cancer to include the possible existence of constitutional factors associated with the entire disease complex. By this concept many changes and events observed in a patient with a malignant tumor are not coincidental findings but are part of a neo-*plasia-oriented syndrome*.[2]

Nieburgs and his group have observed morphologic changes in the nuclei of epithelial and peripheral blood cells from patients with all stages of malignant disease. They have listed rigid, but non-quantitative, criteria for the recognition of these so-called malignancy associated changes (MAC-I). These criteria are a slightly increased nucleocytoplasmic ratio, an orderly structure of the heteropyknotic nuclear chromatin, numerous chromocenters and curved chromatin bands in crowded but orderly arrangements, clear spherical nuclear areas of uniform size surrounded by curved pyknotic chromatin bands, and absence of nucleoli and multinucleated cells. Similar changes were then described later by Nieburgs[4] in gastric smears, liver biopsies, duodenal drainage smears, in the skin of patients with malignant tumors, and in polymorphonuclear leukocytes and lymphocytes of peripheral human capillary blood. Other changes described by Nieburgs as MAC-II changes were observed more frequently in areas adjacent to tumors. Cells which did not fulfill all the criteria of MAC-I or MAC-II were called cells with incomplete or imperfect MAC and their presence is supposed to alert the examiner to watch for cells with the complete or classic MAC, which are less frequently encountered. A good example of a cell nucleus with complete MAC is depicted in Figure 1, while Figure 2 represents incomplete MAC. You will notice that the two pictures differ in that the clear areas in Figure 2 are not completely round or of equal size.

Nieburgs observed MAC-I in smears from the buccal mucosa in 77.2 per cent of cancer patients versus 13.6 per cent of patients without cancer. Using leukocytes of the peripheral blood of cancer patients, Nieburgs and co-workers attained a diagnostic accuracy of up to 88 per cent. Other groups have reported studies of similar nature but with varying results.[1, 3, 5] In this study we attempted to apply Nieburgs' criteria to buccal epithelium in a similar experimental framework and to test the statistical significance of our diagnoses.

MATERIAL AND METHOD

One hundred patients were used for our study. Sixteen had malignant tumors and 15 of these had received no treatment for that disorder. The origin and spread of the tumor in these 16 cases are listed in Table 1. Eight had bronchogenic carcinoma. Cases with oral cancer were excluded from our study. The remaining 84 cases serving as controls were dental students and patients from the complete denture prosthetic section of the dental clinic. The age of the latter subjects approximated that of the cancer group, and in their edentulous state local inflammatory processes were minimal. None of the control patients were on medication nor had they suffered severe or recent illness. The disproportion of our cancer and control cases was used purposely in order to approach better the actual prevalence rates for cancer in the population.

Four firm tongue-blade scrapings, one from each quadrant of the buccal mucosa, were taken from all cancer and control patients. The labeled slides were allowed to air dry, fixed with Spraycyte and then stained by the Papanicolaou method. Thus 400 slides were studied. The slides were randomized and covered for blind reading. The entire series was read three times for the presence of MAC. The second reading was done without knowledge of the previous results. The third reading was done after the results of the first two readings had been analyzed.

Confirmation of the presence of MAC was done at 1000× as minimum magnification. The arrangement of the chromatin bands and chromocenters was studied at this power. Actual measurement of these morphologic changes borders on limits of resolution of the light microscope. For these reasons intranuclear measurements were not feasible. The reading was a subjective interpretation of whether or not the chromatin arrangement seen in the cell nuclei corresponded with that described by Nieburgs as representing MAC. Since the quantity of cells in buccal smears varies, the number of cells on given slide which should show MAC has never been determined. We read a slide as positive if it contained many microscopic fields with incomplete or imperfect MAC and at least one nucleus which fulfilled all criteria for classical MAC, or if three nuclei showed classical MAC criteria without additional incomplete changes.

---

* Presented at the 17th Annual Scientific Meeting of the American Society of Cytology, Chicago, Illinois, November 1969.

RESULTS

A summary of the results analyses are seen in Table II. I these results the number of co was tested in relation to chance distribution based on the controls. The chi-square test was applied to ascertain the significance of the statistical distribution.

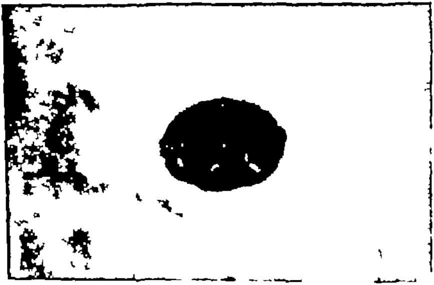

FIG. 1. Classic MAC in cell from patient with bronchogenic carcinoma. The chromatin bands are dense and the nuclear clear spaces are round and free of stain. ×1000.

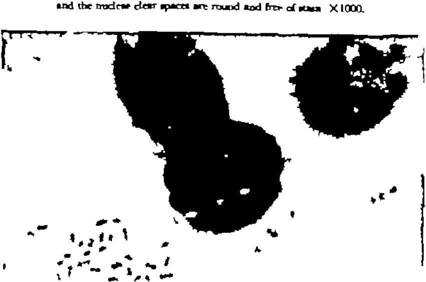

FIG. 2. Incomplete MAC in cell from patient with bronchogenic carcinoma. Note irregular and lightly stained clear areas. ×1000.

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TABLE I *Data of Cancer Cases*

Initials	Age	Sex	Site of primary cancer	Presence and location of metastasis
GG	56	M	Lung	Mediastinum
PR	63	M	Lung	*Supraclavicular nodes, brain*
JA	66	M	Lung	Other lung
HR	71	M	Rectum	Peritoneal implants
CD	63	M	Lung	None
KP	58	M	Lung	Supraclavicular nodes
LG	54	F	Esophagus	Retrogastric lymph node
ED	68	F	Rectum	Regional lymph nodes
RM	54	M	Lung	Mediastinum, brain
BF	45	F	Lung	Brain
SK	23	F	Thyroid	None
MD	50	M	Unknown (und. carcinoma)	Cervical nodes
HB	73	M	Prostate	None
LR	42	F	Rectum	Regional lymph nodes
RD	35	M	Cervical nodes (Hodgkin's)	
RD	42	M	Lung	Widespread

TABLE II *Statistical Analysis of Diagnostic Distribution*

MAC	First reading		Second reading		Third reading	
	Cancer	Control	Cancer	Control	Cancer	Control
Positive	6 (3.36)	15 (17.64)	6 (2.24)	8 (11.76)	8 (14.00)	3 (9.00)
Negative	10 (1[illegible].64)	69 (66.36)	10 (13.76)	76 (72.24)	8 (2.00)	81 (74.00)
Chi^2	3.12		8.74		25.04	
p-value	$< .10$		$< .01$		$< .001$	

Figures in parenthesis represent random expected values.

TABLE III *Accuracy of Diagnosis*

Reading	Cancer cases with MAC		Control cases without MAC	
	Number	% Accuracy	Number	% Accuracy
First	6/16	37.5%	69/84	82.[illegible]%
Second	6/16	37.5%	76/84	90.5%
Third	8/16	50%	81/84	96.5%

The tests gave contradictory probability values. Compared to the conventional 5 per cent "accept or reject" standard, values of 10 per cent were recorded on the first reading, 1 per cent on the second, and 0.1 per cent on the third. In other words, the first test missed significant levels, but the second and third tests indicated that the measured variable, the criteria for MAC, did affect the distribution of the diagnoses.

These values, of course, have no relationship to the diagnostic accuracy as seen in Table III, which shows a maximum diagnostic accuracy of only 50 per cent in spite of our improved skill in differentiating the benign from the malignant cases.

TABLE IV *Reading Agreement*

Cancer cases Positive readings on three readings 3/3	2/3	1/3	0/3
PR	CD	RD	LR
JA		ED	RM
GG		LG	HB
HR		KP	RD
			SK
			BF
			MD

Reading agreement is reflected in Table IV. The initials of the patients are in the appropriate column. The readings in five of the 16 cancer cases varied, but no correlation could be made for any category with the site or spread of the tumor.

Discussion

The subtle variations in MAC and the similarity of MAC with the changes of maturation impair ment make it difficult to use this test as a diagnostic procedure. In addition, we found it impossible to quantitate the number of cells reflecting MAC on any one slide. This is particularly true of the usual buccal smear with its high population of cells. In his original work Nieburgs was also unable to stipulate the number of cells or the number of microscopic fields with complete MAC which he considered necessary for a positive diagnosis since he too found great variations not only in the amount of MAC per case but also per slide in any given case.

Possible explanations for the large number of false negative results in our series are an error by the examiner or actual absence of MAC in buccal smears from cancer patients. In the first instance, inexperience must be considered. The criteria for MAC seem explicit, but the number of cells showing these changes is variable, and the similar morphology of maturation impairment and MAC calls for a very subtle differentiation possible only with experience. The limitation of light microscopy to resolve at the magnification needed for interpretation is also a possible cause for missed diagnoses.

The absence of MAC in buccal smears from cancer patients probably also contributes to the number of false negative diagnoses. Incomplete changes were abundant and continued search for classical MAC in some of these cases was rewarding. Possible explanations for the lack of MAC in cancer patients may include a circadian rhythm effect on mitotic activity or exfoliation, or some obscure tumor-host relationship such as the nutritional status of the patient.

From our findings it must seriously be questioned whether Nieburgs' MAC represent a specific indicator of cancer. The presence of MAC in 15 controls on the first reading, in eight controls on the second reading, and in three controls on the third reading implies that the occurrence of this phenomenon is not limited to cancer. Its association with other conditions was not ruled out by our study.

SUMMARY

Buccal smears of 100 patients were studied for the presence of malignancy associated changes as described by Nieburgs and co-workers. Sixteen of these patients had known cancer and 84 represented normal controls. The four smears taken from each patient were stained by the Papanicolaou method, randomized, and covered for blind reading. The entire series was read three times.

An evaluation of the results suggested that the tested criteria influenced the distribution of the case diagnoses, barely missing significance in one reading but attaining substantial statistical significance in the other two. A reasonable reproducibility of case diagnoses was demonstrated. Because of this we feel that our results permit a cautious acceptance of the MAC concept. However, it is also evident that in our hands the test was too inaccurate for diagnostic purposes.

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Quantitative Criteria of Folate Deficiency in Cervico-Vaginal Cytograms, with Report of a New Parameter

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Folate deficiency is reflected in cervical cytology 8 to 10 weeks before megaloblastosis is clinically evident. Van Niekerk[7] and Kitay[4] have taken various parameters and shown them to be significant. Among these were double nucleation, cytoplasmic vacuolization, nucleo-cytoplasmic ratios, increased cellular size and lobe averages. All of these reflect the disturbed DNA synthesis thought to be associated with folate deficiency while RNA levels remain normal.[3, 7] In attempting to duplicate their findings in both normal clinic and private pregnant, normal non-pregnant, known folate deficient non-pregnant and folate deficient pregnant women, a previously unreported aspect came to light. There is a definite nuclear fold present in the intermediate cells of folate deficient women, whether pregnant or not. Differential counts were performed to quantitate this and render interpretation easier.

MATERIAL AND METHODS

This is a retrospective study. Cervical and vaginal cytograms were compared, and found to be essentially similar, perhaps because the vaginal cytogram contains desquamated cervical cells. The Papanicolaou smears had been prepared in the routine manner, fixed with Carbowax. Differential counts were performed at 400× magnification using initially fields of 500 cells, but as two fields of 100 cells yielded comparable results, this was what was actually used and reported. Categories of patients were: 15 normal women of reproductive age, 5 normal women on birth control pills, 20 clinic and 20 private prenatal patients, 3 pregnant folate deficient women, 20 women with known folate deficiency of whom 12 were premenopausal and 8 postmenopausal.

Peripheral hemoglobin and hematocrit values were available for all patients, but serum folate values for the known folate deficient patients only. Folate was determined by the L. casei method (Spray); levels of 2 mμ or less were considered deficient.

The parameters chosen for comparison were:

1. nuclear folds (Fig. 1)
2. double nucleation of intermediate cells (Fig. 2) and
3. "giant" nuclei (Fig. 3)

Results were expressed in percentages and compared.

RESULTS

Table I lists the results of the differential counts of the four groups, and Table II compares the significance of the values. Folded nuclei were in the same magnitude in the clinic and private prenatal groups, while the difference in double and giant nucleation is significant. Folded, double and giant nucleation was significantly elevated in clinic prenatal and low folate groups.

In the private prenatal group the peripheral hemoglobins were in the range of 11.2 to 13.2 gm/100 ml at the time the smears were obtained. Prenatal supplements had corrected this to over 13 gm/100 ml in all but one case, who had concurrently shown a significantly elevated nuclear fold level. Values were available for 19 or 22 clinic patients both at the time of cytogram and predelivery. Ten had initial values of less than 10.5 gm/100 ml going as low as 8.0 gm/100 ml predelivery while the other nine were over 11.5 gm/100 ml. All these had received iron and multivitamin prenatal supplements which included 1 mg folic acid per day.

DISCUSSION

The work of Van Niekerk and Kitay[3, 4] has linked nuclear changes of the cervical epithelium with folate deficiency. These changes were shown to be reversible by the addition of folate to the diet. It was my good fortune that Dr. W. S. Pepler who is associated with Dr. Van Niekerk, was visiting our medical center and was able to confirm the irregularities of the nuclear membrane and the additional fold which are described and counted in this report. In cervical biopsies of folate deficient women, he found the squamous epithelium atrophic, readily differentiating them from dysplasia. Similar to Pepler's findings, our material

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FIG. 1. Nuclear fold. Cervical smear Papanicolaou stain ×920.

FIG. 2. Double nucleated cell, below nuclear fold in cell above it. Cervical smear Papanicolaou stain ×720.

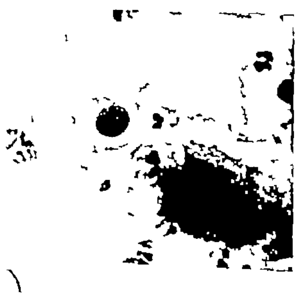

FIG. 3. "Giant nucleus" Cervical smear Papanicolaou stain ×920.

TABLE I *Results of Differential Nuclear Counts (in %)*

Group	*Normal*	*Folded*	*Double*	*Giant*
1 Normal non-pregnant				
Mean	93.5	5.7	0.4	0.4
S E.	0.73	0.65	0.13	0.25
n	39	39	39	39
2 Clinic prenatal patients				
Mean	87.4	6.9	1.9	4.1
S E.	1.05	0.65	0.24	0.76
n	52	52	51	48
3 Known low folate group				
Mean	89.1	4.4	2.8	4.0
S E.	0.81	0.52	0.57	0.72
n	56	56	53	54
4 Private prenatal patients				
Mean	89.5	8.8	0.6	0.9
S E.	0.91	0.83	0.16	0.31
n	44	44	44	44

TABLE II *Significance of Values (p)*

Normal	*Means*		*SE*		*p*
1 vs 2	93.5	87.4	0.73	1.05	<0.001
1 vs 3	93.5	89.1	0.73	0.81	<0.001
2 vs 3	87.4	89.1	1.05	0.81	N.S
4 vs 2	89.5	87.4	0.91	1.05	N.S
Folded					
1 vs 2	5.7	6.9	0.65	0.65	N.S
1 vs 3	5.7	4.4	0.65	0.5	N.S
2 vs 3	6.9	4.4	0.65	0.52	<0.003
4 vs 2	8.8	6.9	0.83	0.65	N.S
Doubl					
1 vs 2	0.4	1.9	0.13	0.24	<0.001
1 vs 3	0.4	2.8	0.13	0.57	<0.001
2 vs 3	1.9	2.8	0.24	0.57	N.S
4 vs 2	0.6	1.9	0.16	0.21	0.001
Giant					
1 vs 2	0.4	4.1	0.25	0.76	<0.001
1 vs 3	0.4	4.0	0.25	0.72	<0.001
2 vs 3	4.1	4.0	0.76	0.72	N.S
4 vs 2	0.9	4.1	0.31	0.76	<0.001

Group 1 Normal nonpregnant premenopausal women

Group 2 Clinic prenatal patients

Group 3 Known low folate group (8 postmenopause) (3 pregnant)

Group 4 Private prenatal patients

has also shown relatively normal epithelia in folate deficient post menopausal women, reflecting the very slow growth rate in the absence of sufficient estrogen.

Folate deficiency has been both blamed for[4] and exonerated from[1] responsibility for preeclampsia, placentation failure and abruptio placentae. No one in our series was toxemic. Correlating our findings with those of Pepler Van Niekerk and Kitay we nevertheless have a way of detecting folate deficiency easily by scrutinizing the cervical and vaginal smears for the above criteria and warning the clinician that more than usual dietary supplementation is in order

SUMMARY AND CONCLUSION

Simple quantitative criteria for detecting folate deficiency in cervical and vaginal cytograms have been presented. The parameters used were nuclear folds, double and giant nucleation. Significant percentages are nuclear folding over 6 per cent, double nucleation 2 per cent, giant nuclei 4 per cent or over Since these changes precede clinical folate deficiency by 8 to 10 weeks, they constitute a valuable aid to the clinician

ACKNOWLEDGMENTS

My sincere thanks to Dr Michael Kyriakos and the staff of the Cytology Laboratory for making their slides available Dr Stuart Kornfeld and Mrs. Carol Goodwin for allowing the use of their folate research series, Dr Walter Wiest for preparing the calculations, the staff of St Louis Maternity Hospital who allowed their patients to be included, and Mrs. Carole Lane for preparing this manuscript.

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Quality Control in Cytology—A Proposed Method for Selection of Slides for Review

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Quality control procedures in the laboratory use replicate analysis as an important tool to detect or prevent error and to provide data for statistical analysis. In cytology, since it is usually impossible to divide a specimen into equal aliquots such replication requires two temporally independent interpretations of the cytosmears from a given case. Federal regulations[1] for licensed cytology laboratories engaged in interstate commerce require rescreening of 10 per cent of the specimens diagnosed as negative and other reports emphasize the need for review of atypical and positive smears[2] and for more extensive programs of quality control.

It seems appropriate to describe a method for the random selection of slides for review. To prevent unconscious bias by the screener, the daily selection of material for rescreening should include atypical and positive as well as negative slides in a variable ratio. The prior dates from which material is selected and the specific accession numbers should also be random. The following report describes a practical method using simple tables of random numbers, which results in a random weighted selection of both normal and non-normal slides. This method is applicable to a cytology laboratory of any size.

REQUIREMENTS

There are three necessary requirements for the random selection method:

1. Sequential numbering of all of the accession numbers.
2. Daily dating of the entry log book to show where each day's accession numbers commence.
3. A retrieval file listing sequentially the accession numbers of all non-negative smears.

OUTLINE OF DAILY MECHANISM TO DETERMINE THE SLIDE NUMBERS

1. Select from a prepared table a random number to determine how many negative and non-negative slides will be examined on this date. The total will always be a constant.
2. Determine a second random number to "back date" the calendar.
3. "Back date" on the calendar and obtain the first accession number on that day.
4. Round the "back dated" accession number to the next lowest thousand to determine a "base number".
5. From the list of non-negative slides, identify and select in sequence the required number of slides whose numbers fall on or after the base accession number obtained in Step 4. All selections must be within the group of 1000 numbers defined by the base number. After all available cases within a group have been reviewed, the entire group can be recycled.
6. From the table of random numbers, add blocks of three digits to the base number until the required selection of normal slides is obtained.

The following discussion and examples illustrate this method. For a hypothetical laboratory with an average daily volume of 100 cytology cases, of which 2 per cent are atypical or positive, the selected goal for a daily quality control review would be an average of ten negative and two atypical and/or positive cases. This average may be obtained by daily review of a total of twelve cases, of which variably eight to twelve should be negative and the remaining zero to four should be non-negative. The laboratory has chosen to select for review material originally screened at a randomly varying interval of three to ten weeks earlier.

The actual slide numbers can be determined as follows. Assume the date of Tuesday, June 2, 1970. Table I, which is derived from a table of random numbers, permits the selection of a variable ratio of negative and non-negative slides. The intersection of the second row (day of month) and second column (day of week) reveals 2(10). This number indicates that two non-negative slides and ten negative slides should be submitted for the day's program. Table II, which has a similar derivation, defines the variable preceeding time interval from which the review material will be selected. In actual practice, Tables I and II can be combined for convenience as in Table III. The intersection of Tuesday, the second day of the month reveals the number 5. By counting backwards on the cal-

TABLE I *Number of Positive and Negative Slides to be Selected*

This partial table indicates the number of positive and negative slides to be selected on any given day The numbers preceeding the parenthesis range from 0 to 4 and are derived from a table of random numbers. The numbers within the parentheses are determined by subtracting the random numbers from a constant, in this instance 12.

Day of Month	Monday # of Slides +	−	Tuesday of Slides +	−	Friday of Slides +	−
1	2	(10)	1	(11)	0	(12)
2	3	(9)	2	(10)	3	(9)
3	3	(9)	2	(10)	4	(8)
31	1	(11)	3	(9)	2	(10)

TABLE II

This partial table indicates the number of weeks, which is used to "back date" the calendar to determine the "base accession" number The numbers range from 3 to 10 and are derived from a table of random numbers.

Day of Month	Monday of Weeks	Tuesday of Weeks	Friday of Weeks
1	7	3	3
2	8	5	7
3	3	3	5
31	10	8	9

endar five weeks, the date of April 28, 1970 is determined The first accession in the log on that day was C-70-9688 Then, from the non-negative retrieval file, select the required number in this case two of non-negative slides, whose numbers occur on or after the base number C-70-9000

The accession numbers of the negative slides are created by adding three-digit random numbers to base number The base number is created by discarding the last three digits of the back dated number and rounding to the next lowest thousand (i e., C-70-9000) Select from Table IV which provides sequential groups of random numbers, the number of three-digit units required to provide for the daily need of negative slides In our example, the accession numbers of the ten additional cases for review therefore, become C-70-9721 C-70-9377 C-70-9385, C-70-9032, C-70-9733 C-70-9353 C-70-9218, C-70-9064 C-70-9739 and finally C-70-9713 The next three-digit number in the table, 610, is the first number to be used on the following day

At the time of review of the selected slides, the reviewer should have available to him all the demographic and clinical information originally available on the requisition, but should have no knowledge of the original report The reviewer' report should be written and documented in the same format as is customary in the laboratory so that a detailed comparison of the initial and of the reviewed report can be performed

DISCUSSION

Each laboratory utilizing its own statistics may modify the scale or timing of this selection method for review slides For convenience, the illustration given is based on laboratory volume of 100 cases

TABLE III *Composite of Table I and II*

This table combines data in Tables I and II for convenience of the user

Day of Month	Monday of Slides/Weeks +	−		Tuesday of Slides/Weeks +	−		Friday of Slides/Weeks +	−	
1	2	(10)	7	1	(11)	3	0	(12)	3
2	3	(9)	8	2	(10)	5	3	(9)	7
3	3	(9)	3	2	(10)	3	4	(8)	5
31	1	(11)	10	3	(9)	8	2	(10)	9

TABLE IV *Random Numbers*

This table of random numbers illustrates the method of selection of three digit groups which are added to the base number to create the specific accession numbers of slides selected for review

72137	73850	32733	35321	80647	39713	61050	57863	65834

per day or approximately 25,000 cases per year The daily volume of twelve is planned to yield an approximate average of ten (10%) negative and two (2%) non negative cases for review It is recognized that the mechanics described for the selection of the "negative slides" will include a small percentage of non-negative slides in proportion to their incidence in the laboratory material On the basis of the data given in the observation, this error would only amount to 2 per cent of the 10 per cent and is considered insignificant.

Laboratories screening more than 25,000 cases per year could most conveniently increase the number of cases selected in proportion to their volume Smaller laboratories could either scale down in proportion or could activate the method described only on those dates on which they complete a block of 100 accessions The method described provides a practical solution to the problems posed by the selection of cytology cases for quality control review The admixture of negative and non-negative material in a random ratio from a variable prior time helps to prevent bias of the reviewer Bias may be further prevented by providing the reviewer the pertinent demographic and clinical information available at the initial screening and by witholding the original report until a second written interpretation is recorded after the review

Evaluation of laboratory performance requires data measuring the variability of performance of individuals and of the laboratory Statistically significant differences between groups depend upon the numerical value of the quotient derived from observed differences between groups divided by a measure of the random error or of variability Without knowledge of this denominator the error or variability one cannot determine whether observed differences between individuals or laboratories have any significance

Valuable data can be obtained from comparative analysis of two independently recorded cytologic reports. At the level of the individual case, the review and comparison offers an opportunity of correction of error and revision of the report for better service to the patient It also provides an important educational focus for identification and solution of problems in technique, in criteria, and in terminology

A series of such reports would provide statistical information in evaluating laboratory performance depending on the completeness of the detail of the report Analysis could reveal the false negative rate and other measures of variability not only for the entire laboratory but for individuals. Indeed, a related quality control program with provision of an opportunity for each individual to serve as reviewer for his own primary reports could yield important data on the day to-day or random variability of individuals.

SUMMARY

1. A method for random selection of cytology slides for quality control review eliminates possible bias by the reviewer
2. The method is designed with flexibility to review 10 per cent of the initially negative and a majority of the non-negative slides in the cytology laboratory

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Vulvar Cytology

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Cytologic studies of the vaginal and urinary mucosae for the evaluation of the sex steroid milieu have well-established practical clinical value The cytologic changes reflect either endogenous hormonal changes during the menstrual cycle or exogenously administered hormones

The cytologic study of smears taken from the upper vagina (colpocytogram) has been the subject of classical observations [illegible] This technique is generally accepted as simple, reliable, and sensitive clinical method of evaluation of ovarian function Despite the present availability of more precise quantitative measurements of urinary and blood hormone levels, cytologic studies are still useful and economical for the rapid assessment of ovarian activity as well as for the study of hormonal production during pregnancy

Hormone-dependent cytologic variations similar to those described in the vagina have also been observed in the epithelium of the urethra and bladder (urocytogram) [illegible] 14 as well as in the rectal, buccal, and nasal mucosae, although these other sites have been shown to be less sensitive target organs than the vagina

In the present study new hormone-sensitive type of cytology is analyzed in order to determine its usefulness and sensitivity as compared to the vaginal smear This report deals with the vulvocytogram, which is the analysis of the desquamated cells obtained from the inner aspect of the labia minora The first observations on cyclic changes in vulvar cytology were reported by Figueroa-Casas et al

MATERIALS AND METHODS

Vaginal and vulvar smears were obtained from ten women whose ages ranged between 27 and 42 years Of this group eight had normal ovulatory cycles, according to concomitant study of vaginal smears, basal body temperature charts (BBT) and cervical mucus changes, one patient had monophasic cycle induced by the administration of an oral long acting estrogen (Quinestrol) and the last menstrual cycle was that of patient in whom ovulation was induced by clomiphene citrate (Clomid**) which is reported to have an anti estrogenic effect in animal studies

The smears were taken two to three times a week throughout one or two complete menstrual cycles In each case samples were taken with Ayre spatulas both from the upper lateral vaginal wall and from the internal surface of the labia minora (nymphae) The material obtained was spread on the surface of clean glass slide immediately fixed in ethanol ether (1 1) and stained by the Harris-Shorr technique The cell population was evaluated by a count of 400 cells in different fields A total of 104 vulvar smears and 104 vaginal smears were processed in eleven menstrual cycles, with an average of nine observations per cycle

RESULTS

Vulvar cytology could not be morphologically distinguished from vaginal cytology except for the smaller number of cells found in the vulvar smear The numbers of superficial eosinophilic cells were counted in both types of smears Comparisons of vaginal and vulvar acidophilic indices are shown in Figures 1 to 4

As expected, the percentage of superficial eosinophilic cells in the vulvar smear was at its highest value near the ovulatory day ranging between 35 and 70 per cent, with mean of 55 8 per cent and median of 60 per cent (±13 1) These values were parallel to, but slightly lower than, the findings for the vaginal cytology which showed a range of 45 to 75 per cent, with a mean value of 65 per cent and a median of 60 per cent (±10 4)

In nine cycles, two of which were in the same patient, the highest per cent of superficial eosinophilic cells in both types of smears occurred on the same cycle day as shown in Figure 1 In the other two cycles the eosinophilic peaks occurred in two consecutive smears, as shown in Figure 2

In the vulvar smears, the lowest values for eosinophilia were found at the beginning and at the end of the menstrual cycle with extremes between 2 and 25 per cent of superficial eosinophilic cells and an average of 12 2 per cent, vaginal cytology was comparable oscillating between 1 and 30 per cent with an average of 12 0 per cent The

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3-cyclopentyl ether of ethynylestradiol Supplied by Warner-Lambert Research Institute Medical Division, Morris Plains, N J

2-[p-(2-chloro-1 2-diphenylvinyl)phenoxy] triethylamine dihydrogen citrate Supplied by William S Merrell Company Cincinnati, Ohio per day or approximately 25,000 cases per year The daily volume of twelve is planned to yield an approximate average of ten (10%) negative and two (2%) non-negative cases for review It is recognized that the mechanics described for the selection of the "negative slides" will include a small percentage of non negative slides in proportion to their incidence in the laboratory material. On the basis of the data given in the observation, this error would only amount to 2 per cent of the 10 per cent and is considered insignificant.

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Valuable data can be obtained from comparative analysis of two independently recorded cytologic reports. At the level of the individual case, the review and comparison offers an opportunity of correction of error and revision of the report for better service to the patient. It also provides an important educational focus for identification and solution of problems in technique in criteria, and in terminology

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SUMMARY

1 A method for random selection of cytology slides for quality control review eliminates possible bias by the reviewer
2. The method is designed with flexibility to review 10 per cent of the initially negative and a majority of the non-negative slides in the cytology laboratory

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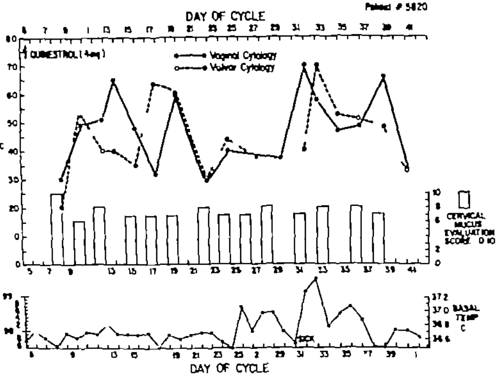

Fig. 3. Vaginal and Vulvar Eosinophilic Indices, Patient # 5820

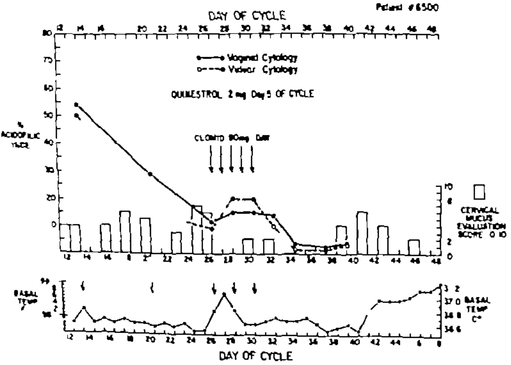

Fig. 4. Vaginal and Vulvar Eosinophilic Indices, Patient # 6500.

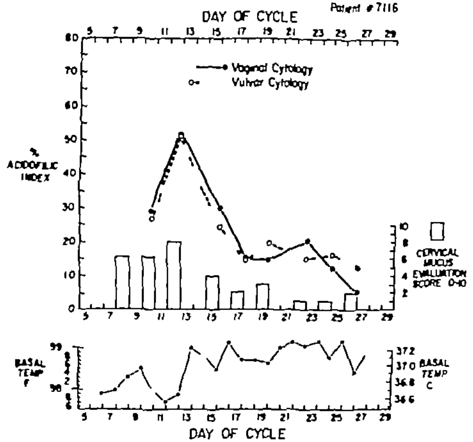

FIG. 1 Vaginal and Vulvar Eosinophilic Indices, Patient #7116.

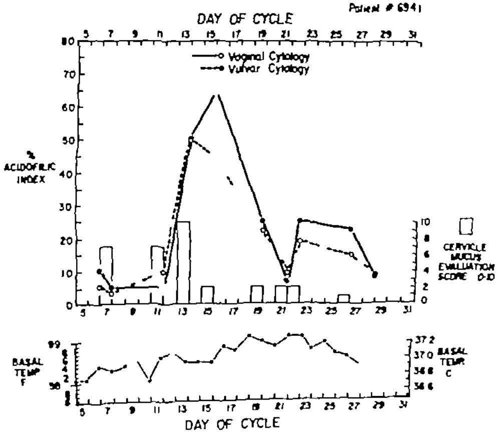

FIG. 2 Vaginal and Vulvar Eosinophilic Indices, Patient #6941

(Quinestrol) showed a concentration of superficial eosinophilic vulvar cells that ranged between 20 and 70 per cent, with an irregular curve essentially similar to the vaginal cytogram, although the points of maximal trophic effect did not correspond exactly (Figs 3 and 9)

The patient on clomiphene citrate therapy showed a slight increase in the number of super

Fig 7 Progestational Phase Vaginal Smear Day 25 Patient #7122 (× 40).

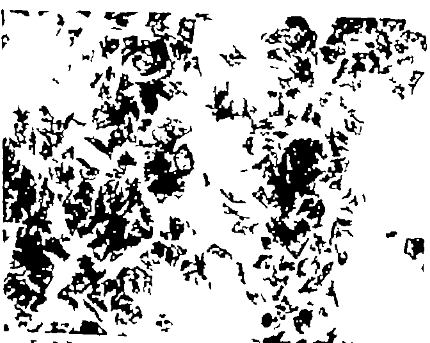

Fig. 8. Progestational Phase Vulvar Smear Day 3 Patient 7122 (× 40)

day of the menstrual cycle which had the lowest percentage of eosinophilic superficial cells for both vaginal and vulvar smears coincided in seven cycles. In six cycles there was a moderate rise in the number of eosinophilic cells in the middle of the luteal phase, producing a second eosinophilic peak lower than the ovulation peak corresponding to the second surge of estrogens during the luteal phase as shown in Figures 1 and 2.

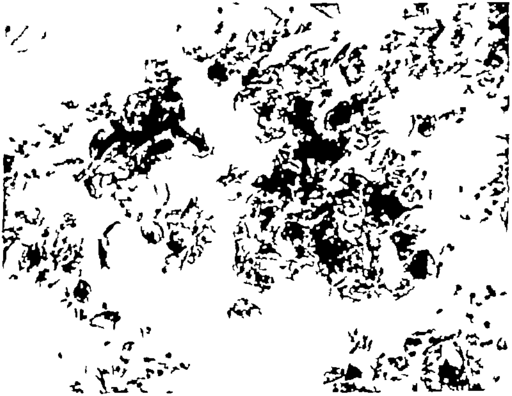

Fig. 5. Ovulatory Vaginal Smear Day 13 Patient #7060 (×240)

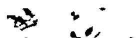

Fig. 6. Ovulatory Vulvar Smear Day 13, Patient #7060 (×240)

The patient under exogenous estrogen effect

(Quinestrol) showed concentration of superficial eosinophilic vulvar cells that ranged between 20 and 70 per cent, with an irregular curve essentially similar to the vaginal cytogram, although the points of maximal trophic effect did not correspond exactly (Figs. 3 and 9)

The patient on clomiphene citrate therapy showed a slight increase in the number of super

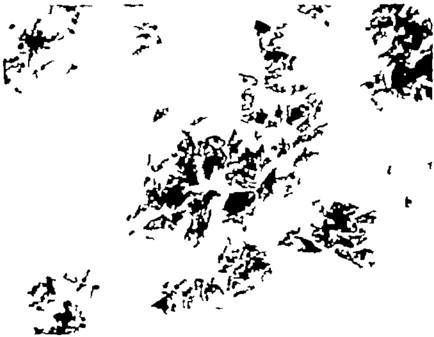

Fig. 7 Progestational Phase Vaginal Smear Day 25, Patient #7122 (×240)

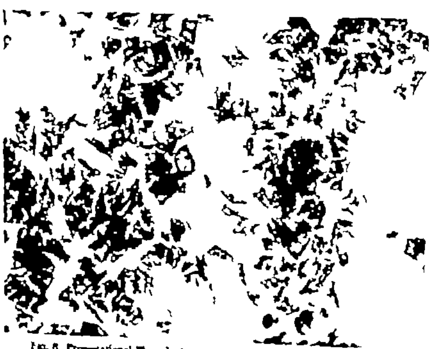

Fig. 8. Progestational Phase Vulvar Smear Day 23 Patient 7122 (×240)

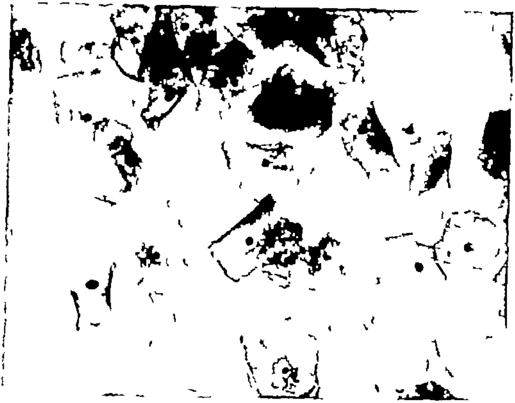

FIG. 9. Vulvar Smear Day 17 Patient #5820 Quinestrol 4 mg administered on Day 5 (X480)

ficial eosinophilic cells during the days on medication. Values decreased in the subsequent three specimens to 1 5 per cent, 1 per cent, and 3 per cent respectively (Fig 4) The colpocytogram showed a similar picture

Qualitative Modification

Both vaginal and vulvar cytologies had identical characteristics During the fertile period or under the effect of estrogen, the smears appeared clear and clean with clearly outlined well separated cells presenting little debris and few leukocytes (Figs 5 and 6)

During the progestational phase of each cycle shedding was abundant, with clusters of cells predominantly of the intermediate type showing irregularity and folding of the cellular edges, mucus, and sometimes bacteria and many leukocytes (Figs. 7 and 8)

DISCUSSION

The studies of nine menstrual cycles in eight ovulatory women, plus the cytologic study in a patient under the influence of a long acting estrogen, and in another patient after the administration of clomiphene citrate, revealed a close parallel between vaginal and vulvar cytology Both epithelia reacted identically with quantitative and qualitative changes. During the fertile period, the percentage of eosinophilic cells was slightly lower in the vulvocytogram, but in this short series the difference was not significant

According to these findings, vulvar cytology seems to be as specific and sensitive a method as vaginal cytology for the qualitative study of hormonal changes in the menstrual cycle or for evaluation of the effect of administered sexual hormones. It may also be useful in the evaluation and management of threatened pregnancies A study of vulvar cytology might be preferable to vaginal cytology in cases of virginity vaginal infection, vaginal bleeding and, of course vaginal aplasia. *In some instances, it might be preferable in cases* of endocrine tumors in children.

Vulvar smears can be taken daily by the patient herself with greater ease, less resistance, and more reliability than those taken from the upper vagina. The interpretation of the vulvar smear is less likely to be affected by the use of vaginal jellies or creams or any other topical contraceptive. Vulvar cytology provides further evidence of the sensitivity with which the mucosal epithelia react to slight sex hormonal changes

SUMMARY

Ten women were studied throughout a menstrual cycle simultaneously by vulvar and vaginal cytology Eight of them had normal ovulatory menstrual cycles one patient received a long acting estrogen which suppressed ovulation and the tenth was studied in a cycle under clomiphene citrate In all ten patients, the qualitative and quantitative cyclic variations of both vaginal and vulvar smears followed the same patterns The reliability of the vulvocytogram as an index of ovarian activity and also in studying the effects of exogenous sex steroids on hormone-sensitive mucosae is established and its possible clinical indications discussed.

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EDITORIAL NOTICE

Automated Cytology a Symposium by correspondence, is an open Symposium All scientists known to work in this area or having interest and plans to study a facet of automated cytology have been invited Also, the scientific community at large is invited to submit their written comments at any time Since the publication of invited remarks and comments will continue at least over the next year there is no deadline for such comments on already published material and material appearing in future issues of *Acta Cytologica*.

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NEWS FROM SOCIETIES OF CYTOLOGY

The European Federation of Cytology Societies

President	Dr. Erica Wachtel, London
Past President	Dr. Jan Zidovsky, Praha
President Elect	Dr. Gabor Sass Dobray, Budapest
Secretary General	Dr. Jacques Jenny, Zürich
Meetings Secretary	Dr. O. A. N. Husain, London.
Treasurer	Dr. Paul Lopes-Cardozo, Leiden.
Treasurer	Dr. Werner Ebert, Jena
Generalsekretariat	Dr. Jacques Jenny Plattenstrasse 51, Ch-8032 Zürich

Asociacion Mexicana de Citologia Exfoliativa

The fifth national congress of Asociación Mexicana de Citología Exf. is going to be held at Zacatecas, Zac., México from October 31 to November 2, 1971. For further details write to:

Asociacion Mexicana de Citologia Exfoliativa
Av. Niños Heroes # 151
México 7, D. F.

Sociedade Latino-americana de Citology

PRESIDENT

Dulce Castellar
Av. N. S. Copacabana 1072/707
Rio de Janeiro-GB-Brasil

VICE PRESIDENT

Hena Cordova De Herrera
Honduras

SECRETARY

Nisio Marcondes Fonseca
Instituto de *Ginecologia da* Universidade Federal do Rio de Janeiro.
Rua Moncorvo *Filho*, 90
Rio de Janeiro-GB-Brasil

TREASURER

Merces Pinto Cunha
Rua Pacifico dos Santos, 63/103
Recife-Pernambuco-Brasil

VOTERS

Patricia Alonso
México

Garcia Giesmann
Peru

Leo Lencioni
Argentina

Zenon Gonzales
Paraguai

The Society headquarters was transferred to Rio de Janeiro, and it is located at the Maternity Institute of the Medicine *and* Surgery School of Rio de Janeiro, in a room donated by Professor Jorge de Rezende.

The 4th Latin American Congress of Cytology will take place on July 1973 in Río de Janeiro.

For information, please contact
Dra. Dulce Castellar
A N S Copacabana, 1072/707
Rio de Janeiro-GB-Brasil

Sociedad Latinoamericana de Citología
Ejercicio 1966–1970

Memoria

1 Comisión Directiva.

La Asamblea realizada en Lima, Perú, durante el II Congreso Latinoamericano de Citología (Noviembre, 1966) resolvió que la Comisión Directiva de la Sociedad quedara constituída en la siguiente forma

Presidente	Dr Guillermo Terzano (Argentina)
Vicepresidente	Dra María Rivas Ros (Venezuela)
Secretario	Dr Leo Lencioni (Argentina)
Tesorero	Dr Ernesto H Bernasconi (Argentina)
Vocales	Dra. Blanca Conde (México)
	Dr Nulo Marcondes Fonseca (Brasil)

Desde entonces los miembros argentinos se reunieron en 38 oportunidades, consultando periódicamente por correspondencia a los miembros de Brasil, México y Venezuela sobre diversos asuntos relacionados con la marcha de la Sociedad la redacción de estatutos, la preparación del III Congreso, etc

La Comisión Directiva realizó su primera reunión plenaria en Río de Janeiro, Brasil, en Mayo de 1968

2 Reunion Extraordinaria

En la ciudad de Río de Janeiro, Brasil, dos días antes de la inauguración del III Congreso Internacional de Citología (Mayo, 1968) la Sociedad realizó una reunión extraordinaria con el fin de discutir temas y problemas considerados entonces de interés para los citólogos de América Latina Se aprovechó esta oportunidad para estrechar vínculos, renovar antiguas amistades y crear nuevas, al mismo tiempo que se estableció un intercambio de opiniones relacionadas con información científica y problemas de la Sociedad

El programa científico se cumplió en la siguiente forma

Viernes 17 de Mayo de 1968

9 30 a 11 30 hs	Mesa redonda Tema ¿Qué es un citólogo?
17 00 a 19 30 hs	Mesa redonda Tema Ubicación del Laboratorio de Citodiagnóstico en un Hospital General
20 00 a 21 30 hs	Sesión de negocios
22 00 hs	Cena de camaradería

Fueron relatores las Doctoras Julieta Calderón de Laguna y Blanca Conde (ambas de México) integrando las mesas redondas los Doctores Ernesto Bernasconi, Ricardo Collillas Marcos Peluffo y Gustavo A Schickendantz, de Argentina, Dulce Castellar Nilo Pereira Luz, María das Mercedes Pontes Cuhna y Victor Valeri, de Brasil Hena Cardona de Herrera, de Honduras, Patricia Alonso y Josefina Parra, de México, Juan García Giesmann, de Peru y María Rivas Ros, de Venezuela

3 Estatutos De La Sociedad

La redacción de los estatutos se fundamentó en las ideas y conceptos expuestos por diversos delegados y/o miembros de la Sociedad y en las respuestas a las consultas formuladas a los ex presidentes de la Sociedad Latinoamericana y a los presidentes de las Sociedades Nacionales en Argentina Brasil y México. Teniendo en cuenta las diferentes opiniones recogidas, la Comisión Directiva elaboró unos estatutos y consideró la conveniencia de ponerlos en vigencia en forma provisoria hasta su aprobación definitiva por la Asamblea Extraordinaria que tendrá lugar en Mar del Plata (11 de octubre de 1970) durante el III Congreso de la Sociedad

El anteproyecto de los estatutos fue enviado para su revisación comentarios y enmiendas a la tres sociedades nacionales. Las diversas modificaciones propuestas a vuelta de correo se aceptaron e incorporaron

4 Conscripcion De Socios.

La Comisión Directiva se abocó de inmediato a la conscripción de nuevos socios, con el objeto de cumplir con las finalidades de la Sociedad que son propender al intercambio científico entre médicos citólogos de América Latina, al mismo tiempo que estimular la unión y la mutua cooperación entre las Sociedades Nacionales de diversos países.

Para lograrlo se invitó a solicitar su ingreso a los miembros de las sociedades nacionales, y en los países en los cuales no existe todavía una sociedad nacional de citología, a los médicos citólogos que acreditaran su idoneidad (se procedió de acuerdo con lo estipulado en los estatutos provisorios)

5 Actualizacion Del Fichero

Se actualizó el fichero de la sociedad Hasta la fecha la sociedad tiene 95 miembros

Titulares	Argentina	38
	Brasil	6
	Costa Rica	1
	Honduras	1
	México	12
	Paraguay	1
	Perú	2
	Venezuela	1
Adherentes	Argentina	25
	México	3
Citotecnólogos	Argentina	2
	Colombia	1
	Costa Rica	1
	México	1
Total		95
Por países	Argentina	65
	Brasil	6
	Colombia	1

Costa Rica	2
Honduras	1
México	16
Paraguay	1
Peru	2
Venezuela	1
Total	93

6 Tercer Congreso Latinoamericano De Citología

De acuerdo con lo dispuesto por la Asamblea (Lima, Peru, Noviembre de 1966) correspondió a la Comisión Directiva organizar el III Congreso Latinoamericano.

Las tareas de organización se iniciaron con la confección de un cuestionario que fue remitido a los ex-presidentes de la Sociedad a los presidentes de las sociedades nacionales de citología y a un grupo de médicos citólogos de reconocido prestigio. En dicho cuestionario se solicitaba opinión respecto a los temas a tratar y a las características que debería imprimirse a la reunión Basados en la información acumulada se designó una Subcomisión de Programa Científico, la cual, bajo la presidencia del Doctor Ernesto H Bernasconi, asumió la responsabilidad de elaborar el siguiente programa, en el que se incluyen

1 Dos conferencias magistrales Dr Eduardo De Robertis Ultraestructura y función de la sinapsis" y Doctor Rodolfo Sammartino Epitelios uterinos anómalos en la recién nacida y en la infancia"

2 Siete paneles Citología Ginecológica Oncológica
Nuevos Horizontes en Citología Exfoliativa
Citología Ginecológica Hormonal
Citología del Pulmón
Citología del Tubo Digestivo
Citología del Tubo Urinario
Catastro de la Población

Se invitó a actuar en calidad de presidentes, coordinadores y panelistas a distinguidos especialistas de Argentina Brasil, Honduras, México, Peru, Uruguay y Venezuela

3 Nueve sesiones dedicadas a comunicaciones libres Figuran 94 trabajos científicos

4 Exposición científica Se han anotado 12 expositores.

El Congreso tendrá lugar en los elegantes salones del Hotel Chateau Frontenac de la ciudad de Mar del Plata, Buenos Aires, Argentina, entre el 10 y el 13 de Octubre de 1970 Esta ciudad ha sido elegida sede del congreso en virtud de su belleza turística y el adecuado ambiente de tranquilidad que ofrece para un congreso científico

El Doctor Bernardo A Houssay aceptó presidir la Comisión Honoraria del Congreso, en el que figuran como vice-presidentes los Doctores Julieta Calderón de Laguna, Jorge Campos Rey de Castro, Clarice do Amaral Ferreira, Ricardo Colillas, Dulce Castellar y Patricia Alonso, y como vocales los Doctores Juan Carlos Ahumada, Inés de Allende, Dante Calandra, Carlos Calatroni Raúl Chevalier Humberto Dionisi, Guillermo di Paola, Osvaldo Eguía, Pedro Figueroa Casas, Miguel A. Goñi, Juan León, Ricardo B Mon, Juan P Picena, David Nölting, Roberto M Pinto y Rodolfo Sammartino

Se constituyeron subcomisiones Fueron invitados a integrarlas un grupo de médicos citólogos, quienes trabajaron activamente, siendo la actuación de ellos de grandísimo valor

Presiden las subcomisiones

Dr Ernesto H Bernasconi Programa Científico
Dr Leo J Lencioni Secretaría General
Dr María Mercedes Gamboni Salones de Conferencia
Dr Francisco Laphitz Flores Exposición Científica y Exposición Comercial
Dr Ricardo D Colillas Recepción y Traslados
Dr Gustavo A. Schickendantz Finanzas
Dr Ernesto H Bonofiglio Prensa y Propaganda
Dr Jorge Bouzas Fiestas y Agasajos
Sra Beatriz S de Colillas Agasajo a Damas Acompañantes.

El numero de inscripciones registradas hasta el momento de redactar esta memoria indica el creciente interés por esta especialidad.

Debemos destacar el hecho que la Sociedad Argentina de Citología ha tenido el *simpático gesto solidario de postergar la realización del Simposio de la sociedad*, correspondiente al año 1969 para hacerlo coincidir con el Congreso Latinoamericano Esta adhesión y el espíritu de colaboración demostrado merece nuestro agardecimiento

Durante este período han sido muy cordiales y satisfactorias las relaciones mantenidas con las sociedades nacionales Sociedad Argentina de Citología, Sociedad Brasileira de Citología y Asociación Mexicana de Citología Hubo un permanente intercambio de correspondencia con motivo de consultas, opiniones, informaciones, etc

La Sociedad tiene su representante (editor) ante ACTA CYTOLOGICA la Doctora Julieta Calderón Laguna ACTA CYTOLOGICA, por cortesía de su editor Dr George L. Wied se ha coupado de anunciar el congreso en las páginas de la revista

La Academia Internacional de Citología su presidente Dr Jorge Campos Rey de Castro, y su secretario, Dr Emmerich von Haam, otorgaron el auspicio de la Academia al Congreso de Mar del Plata

7 Cuota Social.

Se instituyó una cuota social igual a diez dólares anuales.

8. Se imprimieron diplomas que fueron enviados a los miembros de la Sociedad

9 Premio

Se instituyó el Premio "Tercer Congreso Latinoamericano de Citología al mejor trabajo presentado en las Sesiones de Communicaciones Libres

La C.D saliente al entregar la S L.A C. a la comisión entrante, desea hacer publico su agradecimiento a todos aquellos que en una forma o en otra han contribuído al desarrollo de la misma *Al mismo tiempo, saluda con especial* afecto a los jóvenes, esperanza de mañana, que se inician en estas disciplinas de trabajo Esta buena lucha no ha terminado, porque todavía no podemos poner punto final al formidable descubrimiento de Papanicolaou. Estamos *obligados* a contribuir con nuestro trabajo y con todas nuestras fuerzas como si en verdad *fuéramos* a lograrlo.

Dr Leo J Lencioni
Secretario

Dr Guillermo Terzano
Presidente

Buenos Aires, 10 de Setiembre de 1970

Sociedad Argentina de Citología
(*Argentinian Society of Cytology*)

En la ciudad de Mar del Plata tuvo lugar desde el 10 al 13 de Octubre el IV Simposio de la Sociedad Argentina de Citología con la presidencia del Doctor Ricardo D Colillas.

La Comisión Directiva hizo coincidir la fecha del Simposio con ell III Congreso de la Sociedad Latinoamericana de Citología, como cooperación al éxito del mismo.

The IV Symposium of the Sociedad Argentina de Citología was held in the city of Mar del Plata from the 10th to the 13th of October 1970 under the chairmanship of Doctor Ricardo D Colillas

The Executive Committee of the Society celebrated their meeting on this date as an act of cooperation to the success of the III Congress of the Sociedad Latinoamericana de Citología

British Society of Clinical Cytology

The next Annual Meeting of the British Society for Clinical Cytology will take place on 23rd September 1971 at the Middlesex Hospital Medical School, Mortimer Street, London, W 1

The programme has not yet been fixed, but will include scientific demonstrations and proffered papers in the morning and a symposium on Endometrial Pathology and a symposium on Colposcopy and a seminar on problem cases in the afternoon The meeting will be followed by the Annual Dinner at the Zoo (no comments, please!)

In view of the Fourth International Congress of Cytology being held in London in 1971 the Society proposes to hold a one-day meeting only in that year

Československá Společnost pro Gynekologickou Cytologii

(*Czechoslovakian Society of Gynecologic Cytology*)

1 /Czechoslovak Society for the gynecological cytology announces, that Dr Jan Židovský C Sc F I.A C was elected First President of the European Federation of Cytology Societies EFCS European Federation of Cytology Societies was founded October 1st in Prague at the occasion of the First Meeting of European Cytologists

2. /Next business meeting of the Czechoslovak Society for Gynecological Cytology will be held 10 January 1971 The possibilities of the participation of Czechoslovakian cytologists at the meetings in Western Switzerland March 1971 and in London IVth International Congress of Cytology May 1971 will be discussed.

médicos citólogos, quienes trabajaron activamente, siendo la actuación de ellos de grandísimo valor

Presiden las subcomisiones

Dr Ernesto H Bernasconi Programa Científico
Dr Leo J Lencioni Secretaría General
Dr María Mercedes Gamboni Salones de Conferencia
Dr Francisco Laphitz Flores Exposición Científica y
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Durante este período han sido muy cordiales y satisfactorias las relaciones mantenidas con las sociedades nacionales Sociedad Argentina de Citología, Sociedad Brasileira de Citología y Asociación Mexicana de Citología Hubo un permanente intercambio de correspondencia con motivo de consultas, opiniones, informaciones, etc

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Dr Leo J Lencioni
Secretario

Dr Guillermo Terzano
Presidente

Buenos Aires, 10 de Setiembre de 1970

Sociedad Espanola de Citologia
(*Spanish Society of Cytology*)

VI CONGRESO NACIONAL DE CITOLOGIA

La Sociedad Española de Citología celebrará del 3 al 5 de Junio su Congreso Nacional. Este tendrá lugar en Sevilla.

Los temas oficiales del Congreso son los siguientes

-Citología mamaria
-Citoquimia
Ultraestructura en ginecología

Se cursarán algunos temas por citólogos especialmente invitados por la Sociedad Española de citología

Además de las lecciones magistrales a cargo de estas relevantes figuras, se admite cualquier tipo de comunicación libre que haga referencia ya sea a los temas oficiales o a cualquier otro tema citológico de interés. Sin embargo, la selección se realizará por la Comisión Científica en caso de que hubiera un excesivo número de comunicaciones libres

En esta contingencia se procuraría organizar Mesas de Discusión en las que participarían los comunicantes libres para que así de la forma más util para los Congresistas se expusieran los puntos de vista de cada uno

El Congreso Nacional de Citología se celebra precisamente despues del Congreso Mundial de Citología, pues la Sociedad Española de Citología vería con mucho gusto el que en su reunión participaran Citólogos de cualquier parte del Mundo.

La inscripción para los citologos que hayan asistido al Congreso de Londres es absolutamente gratuita

Al margen del interés científico del Congreso, nos atrevemos a recordar que Sevilla es una de las ciudades más hermosas de España y situada en un punto privilegiado de la península ibérica como es la región de Andalucia El interés turístico de esta region es sobradamente conocido y los congresistas tendrán ocasión de admirar las bellezas de todo tipo que encierra esta parte de España

Para toda información científica dirigirse al Secretario del Congreso

Dr D Servando Arbolí
Virgen de Loreto, 35
SEVILLA

y para cualquier información relativa a viajes, excursiones, etc, la agencia oficial del Congreso es

Viajes Touring
Córcega 291
BARCELONA

Österreichische Gesellschaft für Zytologie
(*Austrian Society of Cytology*)

Mitgliedervollversammlung der Österr Gesellschaft für *Angewandte Zytologie* am 20 VI 1970 in Wien

Festvortrag von Prof Dr G Wied, Chikago, über ‘Technisch manipulierte Zytologie ” Die lebhafte Diskussion beweist die durch den Vortrag aufgezeigten faszinierenden Aspekte für die Zukunft Nach Tätigkeitsbericht des Sekretärs und Kassenbericht des Kassenführers, Neuwahl des Vorstandes der Gesellschaft Es wurden gewählt zum Präsidenten Prof Dr H J Holzner 1090 Wien, Spitalg 4 zum Sekretär Dr Rudolf Ulm, 1090 Wien Spitalgasse 23 zum Kassenführer Doz. Dr Erich Burghardt, 8010 *Graz Auenbruggerplatz 14 zu Vorstandsmitglieder* Dr Margarete Fischnaller 1140 Wien, Sanatoriumstr 2, Dr Otto Brunner 5010 Salzburg Schrannengasse 2, Dr Robert Berger 9010 Klagenfurt Neuer Platz 5 Prof Dr Walter Kalkschmid 6010 Innsbruck, Anichstr 35, Zu Rechnungsprüfern *Dr Erich Schnetz 1100 Wien, Sandleitung 9/9/9* und Herrn *Dr Helmut Kastner 8010 Graz, Steyrergasse 137/V*

Svensk Förening for Klinisk Cytologi
(*Swedish Society of Clinical Cytology*)

Annual meeting November 27th in Stockholm sponsored by Swedish Society for Medical Sciences

SCIENTIFIC PROGRAM Symposium concerning

A. Diagnosis of metastasis with morphological methods

1	*Nils Söderström*	The internists viewpoints.
2	Sixten Franzén	Hypernephroma
3	Ulf Engzell	Lymph node metastases in the neck region.
4	Sven Dahlgren	Metastases to the lungs.
5	Alf Lundqvist	Metastases to the liver
6	*Sigvard Persson*	*Metastases to the osseous system*
7	Gunnar Moberger	Histopathological viewpoints.

B. The increasing frequency of gonorrhea The role of inadequate anti inflammatory treatment in population screening program for cervical cancer (N Stormby) Panel discussion together with the sections of microbiology and gynecology

BUSINESS AGENDA

1 Proposal concerning the *I.A.C. licensure and registration of laboratories and* cytotechnologists.
2 Information about the European Federation of Clinical Cytologists including election of two *Swedish representatives.*
3 New plan for education of cytotechnologists.

Sociedad Espanola de Citologia
(Spanish Society of Cytology)

VI CONGRESO NACIONAL DE CITOLOGIA

La Sociedad Española de Citología celebrará del 3 al 5 de Junio su Congreso Nacional. Este tendrá lugar en Sevilla.

Los temas oficiales del Congreso son los siguientes

–Citología mamaria.
–Citoquimia
–Ultraestructura en ginecología

Se cursarán algunos temas por citólogos especialmente invitados por la Sociedad Española de citología.

Además de las lecciones magistrales a cargo de estas relevantes figuras, se admite cualquier tipo de comunicación libre que haga referencia ya sea a los temas oficiales o a cualquier otro tema citológico de interés Sin embargo, la selección se realizará por la Comisión Científica en caso de que hubiera un excesivo numero de comunicaciones libres.

En esta contingencia se procuraría organizar Mesas de Discusión en las que participarían los comunicantes libres para que así de la forma más util para los Congresistas se expusieran los puntos de vista de cada uno.

El Congreso Nacional de Citología se celebra precisamente después del Congreso Mundial de Citología, pues la Sociedad Española de Citología vería con mucho gusto el que en su reunión participaran Citólogos de cualquier parte del Mundo

La inscripción para los citologos que hayan asistido al Congreso de Londres es absolutamente gratuita.

Al margen del interés científico del Congreso, nos atrevemos a recordar que Sevilla es una de las ciudades más hermosas de España y situada en un punto privilegiado de la península ibérica, como es la región de Andalucía. El interés turístico de esta región es sobradamente conocido y los congresistas tendrán ocasión de admirar las bellezas de todo tipo que encierra esta parte de España

Para toda información científica dirigirse al Secretario del Congreso

Dr D Servando Arbolí
Virgen de Loreto, 35
SEVILLA

y para cualquier información relativa a viajes, excursiones, etc, la agencia oficial del Congreso es

Viajes Touring
Córcega, 291
BARCELONA

Nippon Rinsho Saibo Gakkai

(*The Japanese Society of Clinical Cytology*)

The Twelfth General Meeting of the Japanese Society of Clinical Cytology will be held on June 26–27 1971 in Osaka Japan, after the 4th International Congress of Cytology in London.

The Scientific Program will include Special Lectures Symposia on (1) Chromatin of tumor cells, and (2) Hormonal influence on carcinogenesis a Slide Conference, and Proffered papers by congress members.

Please request information by writing to

PROFESSOR JUNJI MIZUNO F.I.A.C.
PRESIDENT OF 12TH GENERAL MEETING JAPANESE SOCIETY OF CLINICAL CYTOLOGY
DEPT OF OBSTET & GYNEC.
KANSAI MEDICAL SCHOOL
FUMIZONO-CHO, MORIGUCHI, OSAKA 570,
JAPAN

Schools of Cytotechnology have been established at the Cancer Institute Hospital in Tokyo and at the Center for Adult Diseases in Osaka with the purpose of specialized training in cytology for medical technologists. At present, a six months course is offered. Each school admits about ten students each year and the number of students graduated from the schools has now reached 65.

An authorized examination for cytotechnologists is held under the co-operation of the Japanese Society of Clinical Cytology and the Japanese Society of Clinical Pathology and to those who pass the examination an authorized degree, *SAIBO KENSASHI* (*CYTOTECHNOLOGIST*) is given. The number of *SAIBO KENSASHE* (*J C T*) reached 68.

For the purpose of supervising the activities of cytotechnologists, the Japanese Society of Clinical Cytology adopted the system of Supervising Cytologists. It is necessary for those appointed Supervising Cytologist to have made outstanding contributions in the cytologic field and to pass the slide conference at the Cytology Meeting. When the Supervising Cytologist becomes unable to manage his duties, his certification will be cancelled. At present, 110 Supervising Cytologists have been appointed by the Japanese Society of Clinical Cytology.

The Finnish Society for Clinical Cytology

The Finnish Society for Clinical Cytology assembled twice in 1970. The Society was visited by Dr S. Franzén, Sweden (parotid aspiration cytology) and Dr O. N. A. Husain, London (automated cytology).

At the Annual Meeting in autumn 1970 Dr Kalevi Laurilahti, Tampere was elected President and Dr Eeiko Purola Helsinki Vicepresident. Dr Pirkko Väänänen, Tampere Central Hospital, Tampere was elected Secretary. The Society has today 43 active and 47 passive members.

At present the training of cytotechnicians is being organized in Finland. The intention is to incorporate the training of cytotechnicians in the program of a laboratory school run by the National Board of Occupational Training. At the request of the Board the Society for Clinical Cytology has drawn up a training curriculum. Moreover, a collection of specimens containing all the most important cell types has been set up for training purposes.

It may be mentioned that the cytotechnicians have formed a national Society of Cytotechnicians, the President of which is Miss Marianne Rönnberg. This society has been very active in promoting the plans for organized training of cytotechnicians and in settling questions related to the interests of this occupational group.

December 8th 1970

Canadian Society of Cytology—Société Canadienne de Cytologie

The current Executive of the Canadian Society of Cytology—Société Canadienne de Cytologie is as follows:

CHAIRMAN

Dr Yvan Boivin,
Hôtel-Dieu de Montréal,
3840, rue St Urbain,
Montréal 131, Qué

PAST CHAIRMAN

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Toronto General Hospital,
Toronto, Ontario

VICE-CHAIRMAN

Dr Ann Worth,
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British Columbia Cancer Institute
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Dr Hugh Curry
Ottawa Civic Hospital
1053 Carling A
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Ottawa 3, Ont

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Dalhousie University
Halifax, N S
Dr Howard Steele
Department of Pathology
Kingston General Hospital,
Kingston, Ont

NATIONAL EDITOR FOR ACTA CYTOLOGICA

Dr Alexander Meisels,
Laval University
Quebec Qué

The next annual meeting of the Society will be held in Saskatoon, Saskatchewan, 2–4 June 1971. It will be a joint meeting with the Canadian Association of Pathologists, with the general theme "Drug-induced Lesions." The program will include a panel on Costs and Benefits of Cytologic Screening Programs.

Further information concerning this meeting can be obtained from the Secretary Treasurer Dr H R Curry Ottawa Civic Hospital, Ottawa Ontario.

The Montreal Cytological Society La Société de Cytologie de Montréal, holds a monthly meeting the last Monday of each month at the Royal Edward Chest Hospital, 3650 rue St Urbain, Montreal P Q., at 8 00 PM The program includes a topic of general interest by an invited guest-speaker and discussion of interesting cases.

Cytology meetings are also held in the Department of Pathology Laval University Quebec City at 4 00 PM the third Wednesday of each month Readers of ACTA CYTOLOGICA are cordially welcome to these sessions.

American Society of Cytology

The 18th Annual Scientific Meeting of the American Society of Cytology convened at the Biltmore Hotel, Los Angeles, California on November 4th. The four day program had a registration of 865 One hundred seventy-eight individuals journeyed to the Surfrider Hotel in Waikiki Beach, Honolulu, Hawaii for a reconvened scientific meeting which ended November 11th. The Scientific Program Committee under the chairmanship of Myron R. Melamed and the Local Arrangements Committee, under the chairmanship of C. P Schwinn, provided an exceptional scientific meeting as well as efficient and delightful extracurricular activities for the participants.

OFFICERS

The newly elected officers include

President Stanley F Patten, Jr M D., Ph D Rochester New York
President Elect Myron R Melamed, M D New York, New York
Vice President Charles P Schwinn, M D Los Angeles, California
Secretary-Treasurer Warren R Lang M D Philadelphia, Pennsylvania
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Richard A Malmgren, M D Bethesda, Maryland
Past President Robert H Fennell, Jr., M D Pittsburgh, Pennsylvania
Executive-Secretary Lenore L. Sngari R N., B S. Philadelphia, Pennsylvania

AWARDS

Papanicolaou Award George L. Wied, M.D., Chicago, Illinois
Cytotechnologist of the Year Award Mrs. Grace Durfee, New York, New York
Best Oral Presentation for a Cytotechnologist Award Patricia Ashton, 1501 La Belle Avenue, Ruxton, Maryland 21204

ANNOUNCEMENT OF FUTURE ANNUAL SCIENTIFIC MEETINGS

1971–Washington-Hilton Hotel, Washington, D.C.
November 4–6

1972–Roosevelt Hotel, New Orleans, Louisiana
November 2–4

1973–Salt Palace, Salt Lake City, Utah
November 7–10

1974–Statler Hilton Hotel, New York, New York
November 6–9

Joint Meeting of the Swiss, German and Austrian Societies of Cytology

March 29–April 3, 1971

The Joint Meeting of the three German-language groups will take place in Wengen, near Interlaken, Switzerland, March 29 to April 3, 1971.

The main topics are Physiology and Pathology of the Uterine Cervix, of the Endometrium and the Endocervix.

The meeting will take place in the Palace Hotel Wengen, 3823 Wengen, Switzerland (Tel. No. 036-34612).

For details, write to Dr. Jacques Jenny, Plattenstrasse 51, Zürich 8032, Switzerland.

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ANNOUNCEMENTS

Fourth Tutorial on Clinical Cytology

August 9 to 21, 1971

The International Academy of Cytology in cooperation with the University of Chicago will sponsor the Fourth Tutorial on Clinical Cytology August 8 to 20 1971 The Tutorial will deal with five days of lectures and workshops on gynecologic cytology (August 9–13) followed by four days of lectures and workshops on clinical cytology of other anatomic sites (August 15–18) and by two days of lectures, workshops and demonstrations on automated cytology (August 19–20)

The tutorial is directed by George L. Wied, M.D., Leopold G Koss, M D and James W Reagan, M D In addition to the directors, the Faculty will consist of Drs. Anthony P Amarose, Gunter F Bahr Peter H Bartels, Marluce Bibbo, John K. Frost Emmerich von Haam O A. N Husain, Alexander Meisels, Myron R Melamed Alan B. P Ng, Stanley F Patten, Jr Abraham E. Rakoff Charles P Schwinn Mojmir G Sonek, Lewis B Woolner and others

The registration fee for the full course is $450 00 for the section of gynecologic cytology alone $250 00 for the section on non-gynecologic cytology alone $200 00, and for the section on cytologic automation alone $75 00

For further details and advance registration, write to

Fourth Tutorial on Clinical Cytology
University of Chicago (HM #449)
5841 Maryland Avenue
Chicago, Illinois 60637

(Advance registration is recommended The registrations for the THIRD TUTORIAL ON CLINICAL CYTOLOGY to be held in Chicago, January 18–22 1971 closed 15 weeks prior to the course.)

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of the CITY UNIVERSITY OF NEW YORK

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LETTER TO THE EDITORS

Histochemical Study of Hydroquinone Tetrazolium Reductase Reaction in Plasma Cells

To the Editor

Tranzer and Pearse[1] described hydroquinone tetrazolium reductase reaction with 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) in order to detect ubiquinones (Coenzyme Q). Ubiquinone acts as an intermediate electron transfer agent between hydroquinone and MTT and reduction of the tetrazole occurs with deposition of the black formazan.

We have applied the aforementioned technique to the systematic study of various human lesions in freshly biopsied material or material recently fixed in 10% formalin. After freezing with liquid nitrogen, sections of 10 μ were obtained in the cryostat. These sections were incubated, applying the method indicated above. Control sections received identical treatment except that the hydroquinone solution was replaced by an equal volume of distilled water. Under the light microscope *some intensely stained cells* were observed in some lesions. The formazan precipitate in these cells was even more than that obtained in eosinophil as described by Hernández and Martínez de Morentin using the same technique. The cells

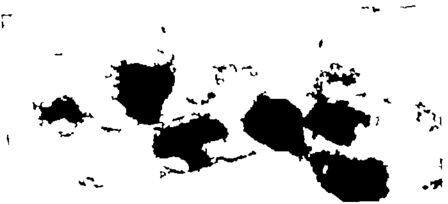

Fig. 1. Hemangioma of skin. Hydroquinone tetrazolium reductase reaction. The cytoplasm of 5 elements intensely stained and appears filled with formazan (×1000).

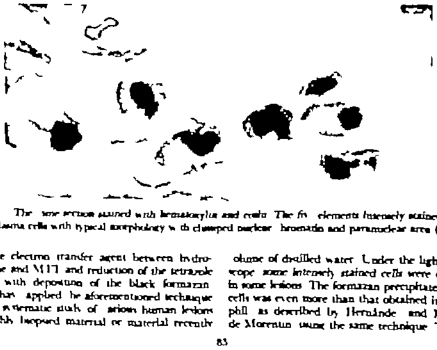

Fig. 2. The *same section stained with hematoxylin and eosin. The five elements intensely stained in Fig.* 1 are plasma cells with typical morphology with clumped nuclear chromatin and paranuclear area (×1000).

were of perivascular disposition, spherical, with eccentric nuclei and a greater intensity of formazan precipitate in the paranuclear region (Fig 1) The reaction was negative in the control sections. We proceeded to remove the coverslips with hot distilled water and to stain the sections where these cells appeared with hematoxylin and eosin (Fig 2) The cells which stained with maximum intensity in the hydroquinone tetrazolium reductase reaction, were found to be plasma cells.

We believe it is of interest to evaluate this great intensity of hydroquinone tetrazolium reductase reaction applied to detect ubiquinones in future investigations of plasma cells. Furthermore the fact that the maximum intensity was in the paranuclear zone, points to a possible connection of the ubiquinones with antibody precursors.

SUMMARY

A histochemical study of ubiquinones on freshly biopsied human material was conducted, utilizing the hydroquinone tetrazolium reductase reaction. In a study of diverse lesions we found some highly positive cells which were identified as plasma cells.

F Hernández, M D and
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REFERENCES

1 Hernández F and Martínez de Morentin, J Cytochemical localization of ubiquinone in human peripheral blood cells. Stain. Tech. 42 241 243, 1967

2. Tranzer J P and Pearse A G E. Cytochemical demonstration of ubiquinones in animal tissues. Nature 199: 1063–1066, 1963.

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AUTOMATED CYTOLOGY

A Symposium by Correspondence

Chapter III Other Parameters, Descriptors to be Considered

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Opening Remarks

Drs. Bartels, Bahr and Wied

Objective Cell Image Description

Cell recognition systems using optical sensing may be grouped into three categories, according to the amount of information carried in the resulting photoelectric signal.

In systems in which the effective measuring aperture is comparable to, or larger in size than the cell the signal consists of a single integrated pulse. Systems of this type shall be referred to as "zero-resolution systems."

In systems which may be termed low resolution devices" the effective measuring aperture has an extension which is small compared to the cell diameter at least in one direction. Typically it consists of a slit, oriented at right angles to the direction of cell transport through the device.

In systems which employ moderat to high resolution scanning the size of the measuring spot is very small compared to total cell area. When digital processing is used, *the cell image is* typically represented by a two-dimensional array of density values. The number of recorded image points may vary from 100 to 500,000.

Systems of the zero— and low resolution design principle are as a rule rather fast and capable of processing samples consisting of large numbers of cells. *They* are designed primarily to render for the measured parameter a value distribution in a very *large population of cells. The intent is the* recognition of this distribution s typical "profile, rather than a recognition of individual cells Occasionally two or three sensors, measuring different cell properties for each passing cell are employed Each cell is then represented by a feature vector of as many components as measures are used *to describe the recorded signal. The* resulting population profile then extends in a two- or *three-dimensional observation space for* example.

In low resolution devices the signal consists of a pulse whose waveform may be used for recognition and classification purposes. Processing may either use the analog signal directly or the signal may be sampled in discrete steps spaced so *as to* give complete representation of the continuous transient waveform. (See discussion of sampling theorem in [14]) In this case, the signal is represented as a sequence of digitized values, consisting typically of a linear sequence of from 10 to 200 sampling points per cell.

Processing of the information at this point of data collection may involve data reduction to a single numerical value or it may extract *descriptors of the signal profile which render* numerical values to such signal properties as pulse height, gradients, percentage of integrated signal falling into portions where the amplitude of the signal assumes a certain value to name a few The cell may thus be characterized by a feature vector of as many components as there are *properties extracted from the signal.*

Simple integration of the signal results in a value representing the integrated relative extinction, also called the "total extinction" in the literature a global cell descriptor frequently used in quantitative cytochemistry (See the general literature survey in) Determination *of the signal duration leads to the global descriptor* relative cell area In any scanning operation spatially consecutive signals appear as a sequence in time. Signal duration may be defined as the number of sampling spots for which the measured amplitude of the signal exceeds a minimum threshold.

In *pulse height discrimination* one may integrate those areas separately where the amplitude of the signal falls into different ranges. In a special case, this leads to the classical cell descriptor "nuclear to cytoplasmic ratio." Combinations of integrated extinction, and relative cell area may be evaluated. This results in the descriptors "average density for the whole cell," "average density for the nucleus," and "average density for the cytoplasm."

The signal may, however, also be subjected to far more complex analysis of its waveform by methods involving spatial frequency filtering and Fourier analysis of the analog signal, as it has found widespread applications in the general field of time series data analysis and pictorial information processing.

Systems rendering highly resolved cell images represent each cell as a two-dimensional array of extinction values (which, when totalled up, form the total extinction), and are, therefore, also referred to as "partial extinction values." Systems of this design principle are aimed at the correct recognition of individual cells, and generally not intended for large through-put. The processing of this signal may involve widely varying degrees of complexity, ranging from a simple integration to decomposition into primitives, processing by special pictorial information computer languages and syntactic analysis.

It has been shown previously that for a typical discrimination problem, the single measured partial extinction value in a digitized cell image carries an amount of information of approximately seven bits. For a typical cell image this means that a total of about 30,000 bits are recorded. Recognition, even in the presence of severe distortion, or image noise, however, requires but 10 to 200 bits, including the required redundancy. A substantial data reduction process is therefore both desirable and necessary.

Image noise, in this context, is composed of several components. Unless very high scanning speeds are employed, photometric noise is almost negligible as compared to the distorting influences introduced by cell sample preparation, and most significantly, pattern variations due to natural variability from cell to cell.

The transmission of the two-dimensional message—as which the cell image prototype of a given cell type may be considered—is therefore affected by numerous distorting influences in the transmission channel. This is represented here by the real world implementation of the image of an idealized cell, its preparation, and subjection to biologic variability. To counteract the effects of this noise, and to enable error free recognition of the signal even in the presence of distorting influences, the encoded cell representation must carry a certain amount of redundancy.

In the extraction of cell image properties, one may distinguish measures which consider the occurrence of each measured extinction value as an event which is independent from the values found in its vicinity. The line scan is considered as coming from a "zero-memory source."[1] The histogram of partial extinction values is an example for such a statistical image descriptor; another is the entropy of the value ensemble, which in essence measures the deviation from equiprobable value distribution.

In the case of the partial extinction values the raw frequencies of occurrence, or the normalized relative frequencies of occurrence may be used, and either the whole histogram, selected bands,[8] or certain linear combinations of the relative frequencies of occurrence from selected bands in the histogram may be utilized to form an image descriptor.

In the first case the Euclidean distance of the whole histogram from the histograms of different cell prototypes may be computed. In the second, differences in histogram contours in suitable regions are exploited.[8] In the third case, the discriminating differences of histogram contour may be optimized. Considering the histogram as a multi-dimensional vector for cells of two different cell types, for example, the vectors representing their histograms occupy different regions in a multi-dimensional observation space. They might be thought of as forming two different point clouds. By removing from the total covariance matrix the contributions resulting from the within-cell-type covariance, the covariance matrix associated with the between-cell type variation can be found, and its eigenvectors. From this, then, those linear combinations of the original relative frequencies of occurrence in the different histogram bands can be found which give maximum discrimination.[19]

While considering each partial extinction value as an independent event is convenient, and in many instances preserves adequate discriminating information, the model is only an approximation of actually existing conditions. Methods which preserve the sequential dependencies of values following each other are in many cases of higher discriminatory value. Analysis of autocorrelation of consecutively measured extinction values along the scan line has shown that an intersymbol influence over from one to five microns may be assumed. The model assumes jointly normal distribution of occurring partial extinction values in a cell population, a condition which is fairly closely approximated. Actually the model of a Markov process[4] is more general, identical for the case of jointly normal distributions, and not identical but more appropriate when this condition is not assumed.

In these data reduction procedures, methods aimed at assessing the "image texture" play an important role. The term "image texture"[11] has been defined as a repetition of well-defined groups of values across the recorded image, or input field. The group constellation can be a random pattern, but the presence of repetition is the significant feature. "Texture" has been used to describe the conditional probability distribution for gray levels in a picture, given the value of the preceding element. Other authors used "texture" to describe two-dimensional covariance and others again mean by "texture" the average size of pictorial elements composing a pattern, a term which is closely related to the number of edges per unit area. Finally, texture is also assessed by measures used to quantitate photographic granularity.[13]

Applying this latter example to the recording of cell images, one may consider that the chromatin granules in the nucleus, for example, appear to be randomly distributed, so that the measured partial extinction values follow a certain distribution. This distribution depends also upon the size of the chosen measuring spot or aperture A. Granularity now is related to the spatial, and amplitude variations of the measured partial extinction values, and assessed by such specific measures as

- *RMS granularity*. This is defined as the standard deviation s of the fluctuation among the measured extinction values.
- *Selwyn granularity*. This is defined as the product of the RMS granularity with the square root of the measuring aperture area, $s\sqrt{A}$.
- *Autocorrelation functions*. Since cellular granularity depends on the sizes of colored cytoplasmic or nuclear granules, the autocorrelation of measured partial extinction values can be examined. The autocorrelation has its maximum value for zero displacement. In the absence of granularity, i.e., constant, and homogeneous extinction over the whole area of the nucleus, this maximum value would apply for any image point. In the presence of granularity, though, the correlation value decreases. Its decrease, expressed as a function of the displacement between two considered points, is a measure for the coarseness of the granules.
- *Power spectrum*. This is the Fourier transform and closely related to the autocorrelation which describes the spatial frequencies present in the recorded cell image.

The biologist can attach more immediate meaning to image properties as they are listed in the following paragraphs. These essentially assess only certain aspects of the more encompassing autocorrelation analysis.

Distribution of Granule Diameters (peak width histogram)[22]

The program examines the sequence of extinction values along both rows and columns of the extinction value array, and determines the distribution of value sequences which rise above a preset threshold, and descend again to values below the threshold, indicating that the scanning spot passed through a granule. The distribution of scan length segments rising above the threshold is a measure of the granule size distribution.

Distribution of Distances from Granule to Granule (peak to peak distance distribution histogram)[22]

In the two-dimensional array the program finds maxima, indicating the presence of densely colored granules. The program forms the distribution of all occurring distances from maximum to maximum both along the rows and columns of the extinction value array. Confined to the area of the nucleus, this analysis records distances of chromatin granules.

Transition Probabilities[4]

Other measures exploiting sequential dependencies are, for example, image descriptors based upon the frequencies of occurrence of transitions from values in certain extinction ranges to values in other ranges, or based upon the probability profiles of such transitions.

When, for example, eight such extinction levels are selected, the program may form an 8 × 8 stochastic matrix for the transition probabilities. The entries here may again be arranged in the form of a linear array, as a profile, and contour differences between profiles obtained from different classes of cell images may be evaluated, and used to form image discriminators following the procedures outlined above for the evaluation of histograms of partial extinctions. One may, however, also use these profiles directly as a template and form "difference patterns" between the profiles derived from an unknown cell, and that of a given prototype.

In this procedure the sequential dependencies extending only over two consecutive measured values are utilized. Methods aimed at evaluating further extending dependencies are, for example, data reduction schemes assessing the covariance between partial extinction values, or other derived quantities representing transformed two-dimensional value arrays.

The covariance matrix permits a decision whether the sequence of extinction values in a cell image could be considered as a stationary time series or not. Preliminary studies on limited material have shown that stationarity seems to be given in images from normal cells, whereas tumor cell images show considerable deviations from stationarity

Techniques which generate patterns involving a substantial number of image points as constellation shall be presented next with few examples

Data reduction begins here frequently with an image clipping in the extreme case reduction to binary state Which image points are to be assigned to state zero and which to state one depends upon the specific algorithm chosen

Examples are provisions which call all image points below or within certain value range zero, all others one or vice versa The gray level representing the cut-off point may be chosen to coincide with the *minimum in the extinction* value histogram This would result in the representation of the nucleus as pattern of dark points The cut-off point may be chosen to extract all values within an extinction value band defined to render the distribution of equiprobable value contours

The same binary encoding may be applied to the derivative of the extinction value array and be used to locate gradients of extinction values In this case the algorithm might specify that all points with difference from their preceding value measured along the line scan, exceeding predetermined threshold, should be assigned to image state one all other points be given the state zero

The clipping operation may be followed by further data reduction, leading eventually to single numerical value Examples, extracted from an image already clipped to binary state are the number of points assigned to state one the dispersion of these points, as measured by such properties as the average distance from point to point the average distance to the nearest neighbor average distance from the center of the image or the number of blocks of four mutually adjoining pattern points

Measures of this type exploit the topographic relationship between image points with certain properties, but in statistical way and not in fashion that would be highly oriented and size dependent Method demanding strict coincidence with prototype constellation, often involving substantial number of image points which must fall into prescribed, mutually dependent value ranges have been employed for cell with their aid, and pronounced morphologic features

Template methods of this type test for coincidence or match, when certain constellation of image points show the demanded properties[18] Either the fact that single match is found in the image or the number of matches are used as classifiers These methods are when applicable highly specific in their ability to discriminate. They are also very inflexible. In this respect they call attention to the difficulties which classification procedures using descriptors as discussed here have in dealing with highly structured patterns

The problems encountered in pattern classification of signals as obtained from low resolution devices have in many instances been solved Suitable techniques are well-established.[14] Patterns of more complex nature such as found in high resolution digitized images have also been successfully discriminated [16, 17] The techniques have in some instances been perfected to where fully automatic programs[4, 18, 19] read the raw data, extract a preliminary set of descriptors for each feature vector component, then select the best of each set, and transgenerate them so as to end up with set of optimized feature vector components For each discrimination problem these are sequenced in an optimized way selected, and applied by other sections of the classification program Cytodiagnostic decisions are made by applying set of decision rules and boundaries, *as automatically determined in the* program

Programs of this type present the present state of the art, and follow in their design rationale the geometric approach This is characterized by the construction of decision boundaries, the extraction of set of image features to form the dimensions of the observation and decision space Most of the image descriptors discussed above are in definition and application, chosen according to this kind of approach The respective programs which are currently applied to real world data will play an important role in the development of automatic cell recognition systems Their self adaptive self-optimizing and pattern detection modes of operation will allow an extension to new pattern classes not considered when the routines were originally designed but for great number of applications their discriminatory ability is already amply sufficient

It appears to be necessary now to study more general systems employing pattern descriptions quite different from what has been discussed so far For reasons already mentioned, one of the inherent problems encountered in the geometric pattern classification schemes is their limited capability to treat complex patterns In single cells the patterns are often sufficiently uniform, and representative in dominant region of the scanned field In tissue sections, one is mostly dealing with patterns of greatly varying texture in diverse image areas

Methods aimed at developing suitable proc aimed at assessing the image texture play an important role. The term image texture[11] has been defined as a repetition of well-defined groups of values across the recorded image or input field. The group constellation can be a random pattern, but the presence of repetition is the significant feature. "Texture" has been used to describe the conditional probability distribution for gray levels in a picture given the value of the preceding element. Other authors used texture to describe two-dimensional covariance, and others again mean by "texture" the average size of pictorial elements composing a pattern, a term which is closely related to the number of edges per unit area. Finally texture is also assessed by measures used to quantitate photographic granularity.[12]

Applying this latter example to the recording of cell images one may consider that the chromatin granules in the nucleus, for example appear to be randomly distributed, so that the measured partial extinction values follow a certain distribution. This distribution depends also upon the *size of the chosen measuring spot, or aperture A.* Granularity now is related to the spatial and amplitude variations of the measured partial extinction values, and assessed by such specific measures as

- *RMS granularity.* This is defined as the standard deviation s of the fluctuation among the measured extinction values.
- *Selwyn granularity.* This is defined as the product of the RMS granularity with the square root of the measuring aperture area, $s\sqrt{A}$.
- *Autocorrelation functions.* Since cellular granularity depends on the sizes of colored cytoplasmic or nuclear granules, the autocorrelation of measured partial extinction values can be examined. The autocorrelation has its maximum value for zero displacement. In the absence of granularity, i.e. constant, and homogeneous extinction over the whole area of the nucleus, this maximum value would apply for any image point. In the presence of granularity though the correlation value decreases. Its decrease, expressed as a function of the displacement between two considered points, is a measure for the coarseness of the granules.
- *Power spectrum.* This is the Fourier transform and closely related to the autocorrelation, which describes the spatial frequencies present in the recorded cell image.

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Methods aimed at developing suitable proc essing techniques for analyzing highly complex patterns are known as the "structural approach" and concentrate on "methods of description."

A pattern is considered as consisting of a number of subpatterns, interrelated according to precisely defined rules, which are also referred to as the "grammar," *a term lent from structural* linguists. Each subpattern in turn may be considered as built up from subpatterns of a lower hierarchy, again interrelated according to the underlying grammar. The hierarchy of stacked levels of pictorial structure may reach into considerable depth. *Finally, the pictorial* pattern is broken down into a set of "primitives" or archetype pictorial elements. These, when combined according to the prescribed grammatical rules form the lowest level of subpatterns.

The structural approach is aimed at having the program develop a set of primitives and two-dimensional concatenation rules, which would allow synthesis of a wide variety of different *pattern* classes.

Pictorial analysis proceeds through a preprocessing stage which records the individual image points from which it must recognize the primitives. The output of the preprocessing consists of a list of primitives and their coordinates in the image. The two-dimensional image is arranged in the form of a linear array listing the interrelated primitives, and subjected to a parsing *operation which examines the interrelationship* between the primitives. The parser output consists of a description of the pattern in terms of its composition of nested subpatterns and primitives, all combined according to the above mentioned rules of concatenation.

Syntactic analysis essentially asks: Is this linear array one of the classes of patterns defined by the rules of two-dimensional concatenation? If so, a description is assigned to the pattern, giving its structure in terms of the underlying grammar. These descriptions are naturally syntax directed, and typically have a tree structure expressing the relationships between the grammatical constituents, *into which the linearly* arranged pictorial information has been analyzed.

As an unknown object is analyzed, syntactic analysis *proceeds through frequent program* checks to assure that the construction fits the description. The decision made at junctures are made according to methods of classical statistical classification theory.

The structural approach has not yet found practical *application* to clinical cell data. Its development is faced with numerous difficulties. The promise of generality and ability to handle highly complex pictorial fields, as well as the inherently high reliability stemming from extensive use of context make syntactical analysis attractive. It can, therefore, be anticipated that cell recognition systems will use methods in the future utilizing both image descriptors formed according to the principles of classical geometric approach, as well as methods following from an analysis of image structure, and its subpatterns.

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Dr Schmidt Weymar

Why Should Coherent Optical Probing Be Applied To Cytodiagnosis?

The search for an automated cytodiagnostic device can be compared with complicated communication problem. A message is to be detected and deciphered, which is given in an unknown range of signal parameters, and in terms of an unknown code. In this comparison, the message is, for example, the malignancy of cell the term signal has to be understood as the physical imprint which is made on communication medium, for example, on the visible light. Assuming that such message is emitted as detectable signal, it can only be read if receiver is available suitable to sense, sample, quantify and process the signal without placing too many active or reactive constraints on the transmitted message. The preparation of the cell may be considered as part of the receiving system.

Decoding and Detection

In order to solve this communication problem, the first approach assumes that the reception of the message is sufficient, the recorded signal is already an analog to the message to be deciphered. In this case, what we are only lacking is the knowledge of the code. For instance, our optical methods of observation may be sufficient in order to evaluate all the parameters necessary to resolve malignancy of cell. We just need to know what, precisely is the clue by which we have to interpret the optical data obtained. The computer of course, has greatly increased our potential to develop an effective working code[1] and, in so doing has enhanced the sensitivity of any receiver by the mere application of its superior memory and combination.

Even by application of the most sophisticated decoding system, if the message received cannot be read satisfactorily one may start pondering whether or not the receiver which is employed has the qualifications necessary for the intended purpose. Since neither the code nor the range of signal parameters of the message to be deciphered is known, one can only guess in which way the receiving system has to be modified. Moreover there does not exist any detector which does not disturb the incoming signal. Are there parameters other than the classical quantitative measures which are likely to yield sensitive, unique, and economic reading of the message of malignancy from cytologic samples?

Optimum Information on the Living Cell

The ideal object to be indentified is one single cell *in situ*, the functional state of which is largely undisturbed. It may be assumed that such an object can be prepared. The probing element may be optical radiation of the visible frequency range. Such an object is characterized by a certain distribution in three spatial and one temporal coordinates of refractive index $(\mathbf{r}, t)$ and absorption coefficient $\alpha(\mathbf{r}, t)$ The ideal optical analysis would have to come up with the complete information given by the numerical data ($(\mathbf{r}, t)$ and $\alpha(\mathbf{r}, t)$ (Such an ideal analysis is not the most economic one which would only contain as little of the complete data as possible in order to allow cell identification.)

It depends on many experimental details of the method of observation how much of the complete information is retained Some more general remarks, however may be suitable for showing which part of the complete information is lost if conventional microscopic techniques are applied.

1 These techniques take the *intensity* of the light arising from the object for quantitative measurements. Hence, the evaluation of the optical image is concerned with absorption or fluorescence by the object rather than with its light scattering properties. Living cells show very little contrast by absorption or fluorescence

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[1] Since the theory of coherent optical scattering to which we implicitly refer in the following considerations, assumes semitransparent, weakly scattering objects, the restriction to visible light is important

staining which is to increase the contrast however commonly changes their functional state.

2. Light *scattering*—commonly found at cytologic objects—is hardly measured by classical optical devices in a quantitative way. The *phase contrast* method displays a *two-dimensional distribution* of the refractive index rather qualitatively and with ambiguity. It does not, in particular detect *the relative phases* of the scattered wavelets. The *interference* transmission microscope measures only the light scattered in the forward direction. The interaction into other directions of wavelets arising from different parts of the scattering object (containing the bulk of information on the structure of the object) is not measured.

3. It is common to all classical cytophotometric methods that for quantitative evaluation, the object is *cut into a large number of isolated elements*. This may be accomplished, for example by optically scanning the object in such a way that only radiation emerging from one small part of the object contributes to the instantaneous signal.

4. Only time averages of the optical constants of the object are considered. This restriction may become questionable if objects *in vitro* are being studied.

I should like to point out that between the existing technical facilities and the object to which they are applied an intense process of mutual adaptation always takes place. For example the living cell has rarely become a cytologic object because of the lack of effective technical facilities to study it. Theoretical and experimental work, however which was stimulated by the invention of the *laser* has shown that new *ways of coherent optical imaging and image processing* are now available, and they should provide the basis for a rather different approach to the microscopic object. How can we use these *new methods to probe cells optically and what advantage would we likely gain by so doing for the purpose of automated cytodiagnosis.*

Coherent Optical Probing

The light beam which emerges from a laser is highly directional, powerful, and extremely monochromatic it possesses an unprecedentedly high degree of spatial coherence. Furthermore, the laser can be "Q-spoiled" in order to emit single giant pulses *of extremely short duration* and very high power and radiance.[9] By virtue of the combination of these features, the laser beam is uniquely suited to make a new approach to cell identification.

First and foremost, the spatial and temporal *coherence of the light waves emerging from the laser* determine the superior quality of laser light. The concept of temporal coherence may be summarized by simply saying that the propagating waves, which carry the optical energy can be pictured in time this is very similar to the well-known sinusoidal oscillation of a.c. current. Moreover the wave fronts (surfaces of equal phase) of the light arising from a laser are precisely shaped so that the phase of the emerging light wave in one point of the plane perpendicular to the optical axis is always precisely related to that of all other points in the same plane[3] (spatial coherence) For example if the laser beam is well collimated, the phase of the emerging wave in one point of the plane perpendicular to the optical axis is precisely the same as in all other points of that plane.

One could never obtain light of such qualifications from a thermal light source.

The remarkable coherence of the laser light could perhaps be utilized to probe microorganisms in ways very different from the ones in use today. The principle of such a procedure would be similar to the "heavy atom method" of X-ray structure analysis[9,10] which has been successfully used in the past to reconstruct in three dimensions the structure of large protein molecules.[11] The basis of this method is the determination of *amplitude* and relative *phase* of the light scattered by the whole object into all directions whereby the mutual interference of the *wavelets arising from different parts of the* object is taken into account (Fig 1). For coherent optical probing amplitude and relative phase would have to be recorded by placing the scattered waves to interference with the reference beam (holography).[13]

The eventual success in employing such a probing technique will depend on three conditions, the first of which is the most serious one.

1. The surfaces of equal phase (wave fronts) of the incident wave must be known over the scattering region *with at least the same degree of* accuracy as the desired spatial resolution (both in the lateral and longitudinal coordinates). This hard condition on the spatial coherence of the illuminating light can, however be met by the laser.[12]

Multiple scattering of the light inside the object should be neglectable *(Rayleigh-Debye* approximation) In order to more conveniently make the numerical evaluation of the scattering data. For visible light, the Rayleigh-Debye

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Instead of sectioning the object plane one could, *in a conventional* optical system, also observe and section the intensity *in the* Fourier transform plane, e.g. in the focal plane of the objective lens. This does not reduce the loss of the information due to sectioning of the image if we do not consider coherent optical illuminating and recording methods.

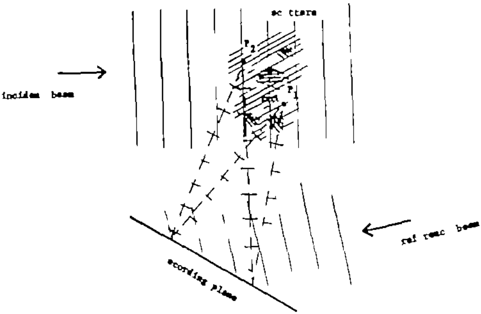

Fig 1 Coherent optical structure analysis

approximation is good within useful range of refractive index, object thickness, and scattering angle The solution of the inverse scattering problem, however is also possible for other systems which do not allow the Rayleigh-Debye approximation treatment [8]

3 In order to make the method quantitative, calibration is necessary This may be done by applying model objects, for example, polystyrene latex spheres or pollen particles, which have well-known scattering characteristics in the range of visible light

Similar considerations applied to the interference in *time* of scattered waves rather than to their superposition in space lead to the conclusion that even relatively small movements in space of scattering objects or small variations of the scattering parameters (refractive index) of the object, can be detected from the spectral analysis of the scattered light. Well-known examples are the accurate measurement of the velocity of a moving sheet of paper moving liquid or gas, using laser light The principle of the method, which measures the Doppler frequency shift, is shown in Figure 2. At present, it seems possible with this method to measure relative velocities down to lower limit of ca 5×10^{-4} cm/sec Applications of the laser Doppler velocimeter to blood flow measurements on exposed vessels have been suggested.

Cytology also, is likely to gain something from the high monochromacy of the laser light

1 Since the velocity of protoplasmic streaming (0 006 mm/min – 60 mm/min) is in the range of the laser Doppler velocimeter protoplasmic motion may be measured.

2 Submicroscopic movements of the macromolecules, which are the building blocks of protoplasmic structures, are likely to be detectable in similar way to the measurement of the diffusion coefficient of suspended polystyrene latex spheres (0 55 μ and 0 088 μ diameter)[9] from high resolution spectral analysis of the Rayleigh scattered laser light It is the intracellular viscosity which can be measured *in vivo* and in real time with this technique

A somewhat more detailed discussion of two potential applications of coherent optical methods to automated cytology is given in the following sections

Cell Structure Analysis in Three Dimensions

The first experimental problem here is to place the scatterer into the scattering area in suitable way (Fig 3) It is required that, during the measuring time

( ) the cells should be separated from each other

(b) the movement of the cells should be sufficiently small in order to avoid disturbance of the interferometrically recorded, scattering data,

( ) anisotropic cells should be oriented relative to the incident beam

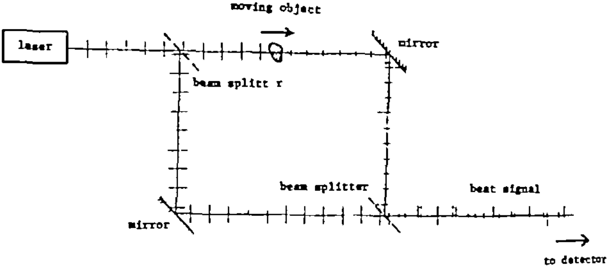

Fig. 2. Laser Doppler effect velocimeter

A laminar nozzle flow chamber of the type recently reported by Mullaney *et al*[11] is likely to satisfy the above conditions (a) and (b) In connection with a pulsed laser this flow system can also meet the condition (b) For a typical nozzle, an exposure time of less than ca 50 nanoseconds is necessary to interferometrically record the scattered waves. A "Q-spoiled" laser delivers sufficient energy within that time interval, while still retaining enough of its spatial and temporal coherence. The laser pulse has to be synchronized with the occurrence of one cell at the measuring spot. Since the cells carried by the sample flow are well separated on the average[1] the synchronization does not cause serious difficulties. Other problems in such a set-up would be the design of a suitable reference source and the techniques of recording the interference fringes. A pinhole in close proximity to the scatterer (possibly of known position with respect to the scatterer) is likely to be the reference source which places the least stringent requirements on the resolution power of the recording material, and this type of reference source would also facilitate the evaluation of the scattering data. Other reference sources may however be considered.[1]

The recording of the interference intensity pattern can be done photographically Emulsions of some 1000 lines/mm resolution power are available From the recorded data, a reconstruction in three dimensions of the optical constants of the scatterer in particular the refractive index, is possible.[4, 11] For this purpose, the photographic plate would have to be scanned by a suitable microphotometer

The pattern arising from the interference between *object* wave and *reference* wave may also be sensed and sampled by an electro-optical image conversion tube. Since the exposure time is too short to allow parallel sampling of the recording plane, a high resolution storage tube such as the Westinghouse Permachon vidicon would have to be employed. For digital computations, the data have to be analog-to-digital converted.

Some variant of this recording principle (Fig 3) may be mentioned. Basically the simple pinhole which acts as the reference source may be *replaced by an optical transparency* such as the hologram of a "reference object" for example, a cell of certain dimensions and given refractive index distribution. (Such a reference hologram, however may be computer synthesized and may correspond to a "synthesized object" rather than to any particular one[9, 14]) Such a coherent optical "matched filter" technique would likely reduce the amount of data to be processed and, perhaps, completely eliminate the scanning of the hologram plane

Intracellular Viscosity and Protoplasmic Streaming

The high resolution spectral analysis of the light scattered from the cytoplasm or in the first instance, from model proteinaceous colloids, seems to be a fairly straightforward procedure. Figure 4 shows, for example, alternate ways of signal processing using a self-beating type of high resolution spectrometer The different methods of signal processing are in order to distinguish between different physical and physiological effects, all of which change the spectral composition of the scattered light Heterodyning methods not discussed here, have additional capabilities[2, 17] for this purpose

Such an instrument allows the measurement of harmonic frequency components contained in the scattered light within the range from ca 1 c/sec to some megacycles/sec. In particular the very low frequency components are of interest.

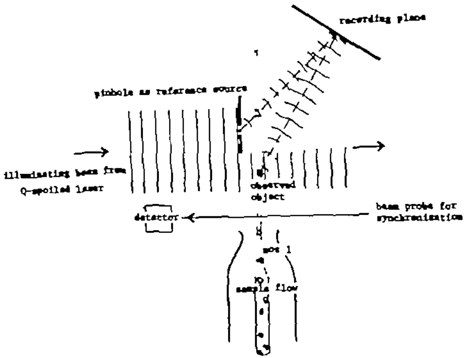

FIG. 3. Schematic set-up for coherent optical probing of suspended cells.

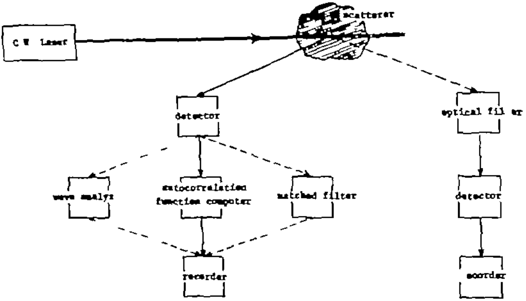

FIG. 4 High resolution spectral analysis of scattered laser light.

For instance the spectral line broadening of the Rayleigh scattered light by the globular protein molecules of the hyaloplasm (100 Å diameter) is ca 5 k /sec. The main experimental problem in this method is how to avoid any acoustical vibrations of the set-up. There are stable optical table systems available which use an effective pneumatic acoustical vibration isolation. The remaining problem is to keep the cells in a fixed position for the time of measurement. This turns out to be a serious problem but it is quite possible to solve it cell movements faster than ca 1 µ per sec—in particular turbulent ones—must be prevented.

Potential for Automated Cytology

Assuming the technical and practical feasibility of such coherent optical devices is given, what could be gained from them for an automated cytological procedure? In summary coherent *optical structure analysis of cells in vitro* could yield quantitative data in three dimensions of the optical scattering of the whole cell. Intra*cellular viscosity of living cells* may be measured in real time from high resolution spectroscopy of quasi-coherent scattered light. In addition, proto*plasmic streaming* may be determined.

The information potentially gained from coherent optical methods, if compared with the "classical" methods, *has two characteristic* features. It is likely to be a more complete set of quantitative data on the object and it is measured *on living cells*.

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DRS. VAN DILLA AND FULWYLER

CELLULAR PARAMETERS MEASURABLE IN FLOW SYSTEMS

In these opening remarks we discuss cellular properties which can be measured in flow systems and examine the sensors which can presently measure the distribution of a single parameter (such as volume or Feulgen-DNA) among the members of a cell population, the possibilities of other sensors, and finally the combination of single sensors into multiple-parameter systems. In flow-through analysis systems, cells pass rapidly through a sensing volume one at a time. Because measurement requires only 20 to 50 µsec and cells are analyzed at rates of 10^4 to 10 /min, detailed morphological information of the type traditionally obtained by microscopic observation is not available. In contrast, flow systems offer *rapid and, of great importance quantitative* analysis of functional and biochemical properties. Statistical precision is very good because *of the large numbers of cells measured.* The more obvious methods of sensing cellular properties have been explored to a limited extent, but it seems certain that other techniques await definition and application.

SINGLE-PARAMETER ANALYSIS

A. *Fluorescence Methods*

The advantages and limitations of microfluo*rometry using the fluorescence micro-scope* have been discussed by Ruch.[17] As applied to flow

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This work was performed under the auspices of the U.S. Atomic Energy Commission.

systems, fluorescence sensing has several advantages over absorption methods generally used in cytophotometry Because a cell can be in any part of the cell stream, the incident light beam must be larger than cell stream Consequently the cell intercepts relatively small fraction of the incident light, and the decrease in total transmitted light is small—much less than is the case in conventional absorption cytophotometry where a stop is used to confine the beam to the cell, minimizing background illumination. In contrast, microfluorometry is a dark field method and only cell fluorescence, modulated by any background fluorescence, is detected, optical contrast is inherently good In addition, error due to nonuniform distribution of the fluorochrome within cell (i distributional error) is generally negligible, thus avoiding complication encountered in absorption cytophotometry

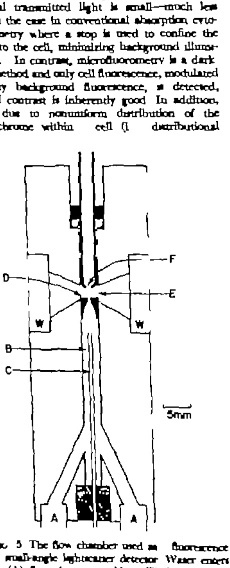

Fig. 5 The flow chamber used as fluorescence and small-angle light-scatter detector Water enters ports (A), flows down central bore (B) is constricted by nozzle (D) and flows as jet across viewing region (E) and out via exit tube (F) The cell suspension is introduced on the flow axis through tube (C) The exciting light from an argon ion laser enters through one of the windows (W) crosses the cell stream, and exits through the other window Fluorescence is sensed through window at 90° to the incident beam and small angle light scattering is detected through the laser beam exit window

1 The fluorescent Feulgen procedure described by Kasten and later used by Ruch has been adapted to cells in suspension by Va Dilla *et al* and is described in more detail by Mullaney *et al.*[13] Using the flow system shown i Figure 5, an argon ion laser to excite fluorescence emission, and multichannel pulse-heigh analyzer to analyze and store the data, Va Dilla *et al* have obtained DNA distributions o exponentially growing tissue-culture cell populations, normal human leukocytes, and cells from other tissues These distributions are quantitative in the relative sense If absolute DNA conten is desired, cells of know DNA content must be present as calibration standard In the case o exponentially growing tissue-culture cells, internal calibration is automatically supplied by the presence of diploid (G_1) cells and tetraploid (G_2 and M) cells. In some cases only the shape of the distribution is important, and there is n calibration problem When calibration is necessary cells with known and appropriate characteristics can be added and carried through the Feulgen procedure For example, if tumor-cel population is suspected of containing excess DNA then normal diploid cells (like lymphocytes can be added to provide a normal diploid calibration peak Sperm cells would provide haploid peak out of the range of interest, but thei staining properties (presumably due to high degree of DNA compaction) makes their us questionable

DNA distributions in several types of human carcinomas have been studied using cytophotometric methods[10] A recent example is study o DNA distribution of bladder tumors by Lev *et al.* The authors are unable to demonstrate th particular ploidy carries good or bad prognosis, but their methods appear practical, yield new information about tumors, and may giv pointers on the widely differing behavior o tumors that have the same clinical and histological appearance W may conclude that th parameter (e., DNA content) is of value i cancer-cell analysis but that it is not sufficien Hopefully future work will clarify its role may be more useful as component in multiple parameter analysis

2 The staining properties of the fluorochrom acridine orange have stimulated considerabl interest in this dye however most of this wor has been qualitative and difficult to evaluate recent study by Rigler[14] suggests that the met chromatic properties of this dye can be used t measure quantitatively both DNA and RN content. Acridine orange binds to double-strande helical DNA in monomeric molecular for

with a fluorescence emission maximum at about 530 nm wavelength (green) With diminishing double-helical content of the nucleic acid molecule the dye binds to an increasing extent in the associated molecular form, allowing interaction between adjacent dye molecules and shifting the emission to longer wavelengths. Thus, the emission peak of the ribosomal RNA-dye complex is at 650 nm wavelength (red) If structural order is completely lost (random coil) there is an even greater shift to the red. *Protein interference with dye binding* can be eliminated by blocking agents and proper choice of pH By using optical filters or color-dispersive elements and two or more photomultiplier tubes to independently sense green and red this method offers the possibility of simultaneous measurement of cell DNA and RNA content.

3 The phenomenon called "fluorochromasia"[14] has been used as an indicator of intracellular enzyme activity When coupled with flow system analysis, it may enable automated analysis of *cell* esterase activity membrane transport function, viability and presence of other enzymes. Live cells incubated with fluorescein diacetate (nonfluorescent) readily take up and hydrolyze this fluorogenic substrate, releasing free fluorescein within the cell. Because fluorescein is not rapidly transported across the cell membrane, an intracellular accumulation occurs, rendering the cell fluorescent. Fluorochromasia correlates with cell viability activity of esterase enzyme,[15] antibody producing ability of mouse spleen cells, and possibly with other functional properties of cells. Use of other fluorogenic substrates to assay other enzyme activities is a potentially powerful technique

The techniques of fluorescently labeled antibodies and antigens are now widely used in diagnosis and research and appear to be adaptable to flow-system analysis. The general principle was described by Coons and Kaplan. Antibody obtained in free, isolated form is conjugated with fluorescein-isothiocyanate yielding fluorescently labeled antibody The corresponding antigen is added to a mixture of cells and attaches to those carrying antibody on their surface. The labeled antibody preparation is added and binds to the attached antigen, yielding fluorescently labeled cells. This technique may be modified to label cells with specific antigenic properties if the corresponding antibody can be prepared and isolated.

4 A variety of fluorescence staining methods used in fluorescence microscopy to obtain information on cellular *proteins* may be useful in flow systems. These include Geranine G for basic protein, brilliant sulfaflavine for total protein, ninhydrin for arginine, and dansylchloride for lysine. Also, *new methods have been appearing in* the recent literature. One of these uses a chelate of europium as a fluorescent stain for bacteria[3] and mammalian cells.[4] Staining occurs mainly in the nuclear material, probably because of formation of insoluble salts of nucleic acids. Another[4] uses DNase conjugated with fluorescein-isothiocyanate as a specific stain for labeling DNA in the cell nucleus. These methods offer possibilities for flow microfluorometry but suitable procedures for cells in suspension will have to be developed.

5 We *have* assumed so *far* that the exciting light beam diameter is much larger than cell diameter so that all cellular detail is lost and only total fluorescence light emission per cell is measured. This is actually the case in current flow systems but needn't be so. One can generate an exciting laser beam of elliptical cross section with axes 2 and 50 μ long If cells cross the beam along the short axis, they are scanned in one dimension and crude structural features may be detectable. Application to cells stained metachromatically with acridine orange or to *differential* counting of leukocytes are intriguing possibilities for the future.

B Light Scatter Methods

The phenomenon of gross light scattering by suspensions of particles of *cellular dimensions* has long been used to measure particle concentration. Methods of detecting single particles have been developed more recently and theoretical treatments have been worked out [21, 22, 23] which make the method attractive as a way of obtaining size and structural information from single cells at high speed The method of Mullaney *et al.* [22] makes use of essentially the same flow system used by Van Dilla *et al.* [18] with the exception that light scattered at small angles (0.5 to 2.0°) to the laser beam is measured. *For cells in the diameter range* 5 to 20 μ suspended in aqueous medium, the amount of such scattered light is approximately proportional to cell volume, yielding an optical method of cell sizing Wyatt[23] has recently used differential light scattering at wide angles for identification of bacteria. These new approaches open exciting possibilities for analysis of cell properties.

C Coulter Volume Methods

Both the theoretical and experimental aspects of electrical sizing of particles in suspension have been subjected recently to critical analysis by Grover *et al.* They discuss the effects of electric and hydrodynamic fields on the orientation and deformation of spheres, migration of particles toward the orifice axis, and principles underlying generation of an electrical signal. Experimental pulse shapes are compared with theoretical predictions and confirm that, under proper conditions, accurate volume distributions of polystyrene latex spheres are obtained. Their work on living cells is now in progress, but previous work in this area has shown the method highly useful. The Coulter transducer has been the most widely used sensor for flow systems, mostly for the purpose of measuring cell concentration (i.e., number of cells per ml of suspension). Accurate volume measurement requires more sophisticated equipment and is subject to errors that must be avoided. The measurement of volume is especially useful when the cell's functional state is reflected by its volume.

D. Light Absorption Methods

Light absorption by stained or unstained cells is the standard method of cytophotometry and is used by Kamentsky *et al.* in their flow system for rapid cell analysis. Absorption at 253.7 nm wavelength gives a measure of nucleic-acid content of unstained cells and light scattered by the cell an estimate of cell size. Staining methods such as the Feulgen procedure for DNA, naphthol yellow S for protein, and trypan blue were later used to characterize other cellular parameters.[24] Unfortunately the absorption method as currently applied to flow systems is difficult to employ because small decrease in a large light signal must be measured however breakthrough here would increase the attractiveness of the method.

E. Possible Extensions of Single-Parameter Analysis

It may be worthwhile to speculate on what might develop in the way of more sophisticated and imaginative methods of cell sensing. Optical methods might be extended to spectroscopy (i.e. fluorescence or absorption as function of wavelength) by use of dispersive elements or dichroic mirrors and many light sensors. The current development of small, low cost, general-purpose digital computers simplifies the data handling problems involved. Natural fluorescence of cellular proteins and other components may become accessible. Use of pulsed-lasers as light sources for fluorescence offers many advantages, including ery high exciting light intensity on the cell during the pulse and no background fluorescence between cells when the exciting light is off. Current work on high-speed laser beam deflectors suggests the possibility of cell scanning in flow systems. Excitation by other radiation than light (for example, by electrons as in X-ray microprobe analysis or by heavy charged particles or neutrons) could yield new information on cellular constituents like sodium, potassium, and phosphorus. If enough prompt radiation were produced per cell, nuclear radiation detector technology—already developed to high state—would be available. Some of these far-out ideas might be more feasible with the cell stream in the form of a free jet rather than confined in a flow chamber. At this stage in evolution of high-speed physical examination techniques, it is obvious that a great deal more information and cross correlations are necessary before the limits and merits of the various methods can be determined. It appears to the authors that the door to this kind of knowledge has been barely opened and that no one can predict what is really going to be found.

MULTIPLE-PARAMETER ANALYSIS

For many purposes, as when one wishes to distinguish several categories of cells found in a mixed population, measurement of a single parameter does not provide adequate discrimination, and the need to measure several cell properties becomes apparent. By moving the cell past sensors, flow systems enable one to perform simultaneous or serial measurement of several properties of each cell. Sensor systems can be combined and, should it be desirable, the signals generated can be processed in various ways to modify their dependence on the measured property. For example, signal proportional to cell volume (r^3) could be processed to make it linearly proportional to cell radius (r) or to area (r^2). Because only one piece of data is produced by each sensor for each cell the amount of information to be processed is small, and the requirements placed on the electronics are not great. By using two-dimensional pulse-height analyzer two-parameter frequency distribution of cells could be obtained. Three- or more parameter analysis would require the data capacity of a small computer. As an alternative to storage of all information, logical restrictions could be imposed on the analysis scheme, thus lessening the electronics requirement. For example, if one wanted the fluorescence distribution of all cells of volume between 300 and 400 μm³ this could be obtained by analyzing the fluorescence of only those cells which had produced a volume signal corresponding to the desired range. Or the reverse question could be asked what is the volume distribution of those cells with fluorescence in selected range? In this case, the fluorescence pulse must have been in the selected range for the volume pulse to be analyzed. If biologically useful, one can imagine analysis on the basis of three or four such logical requirements.

Alternatively the processed signals from several sensors could be combined as ratios (or sums, differences, etc.) and the frequency distribution of this ratio among a population of cells determined. For example, by using RNA-specific fluorescent stain and sensors for fluorescence and volume, one could form ratio of the processed signals to obtain a distribution of RNA [illegible] ing a population of cells. Likewise, a type of nuclear-to-cytoplasmic ratio would be given by using a nucleus-specific fluorescent stain to give a measure of nuclear volume and total cell volume measurement by scattered light or by the Coulter sensor Perhaps quantitative measurement of this property would be of use in identifying cancer cells.

The ability to analyze rapidly and quantitatively a population of cells by the methods discussed might reveal previously unexpected and quite definitive differences among apparently similar cells. To categorize cell types in quantitative terms of DNA-to-RNA ratio, presence or absence of specific enzymes, cell volume, reaction with specific antibody or antigen, reaction with chemically-specific stains, etc. might enable one to develop a new set of classification criteria which would be functionally more specific and meaningful. Hopefully some combination of measured properties could be obtained which would uniquely define malignant cells, permitting automatic identification. Criteria arrived at in this way would be relatively abstract and should be correlated with the current morphologic criteria. This would be best facilitated by electronic sorting which would permit visual examination of cells selected according to electrically or optically measured characteristics.

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DR. YOUNG

OTHER PARAMETERS

In the fields of image processing pattern recognition, electrooptics, and heuristic programming

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technology has improved to the point where we can now consider the design and construction of systems for the automated analysis of visual data. With respect to cytology such systems can play large part in both biological research and clinical medicine. What is clearly needed however is an understanding of the inherent strengths and weaknesses of such devices. To achieve this understanding we must begin by recognizing the mechanisms and parameters by which the cytologist presently manipulates his visual inputs.

Within the contexts of pattern recognition and image processing, it should be clear that the human being is extremely good at decisions involving qualitative judgments. Is the object red? Is it circular? Is it large? However in terms of quantitative judgments—how much "redder" is this sample than that?—our judgment is extremely poor especially when compared to some physical scale or standard.

My particular field of interest in cytology is the automated leukocyte differential and, thus, let me draw upon it for examples. The differentiation among the five different types of normal leukocytes is normally performed not on the cells themselves, but upon the way they look when smeared and stained with Wright' blood stain. The lab technician, intern or clinician sorts the various cell images into appropriate categories by making qualitative observations about the size, color shape and texture of both the nucleus and cytoplasm. One of these parameters, color is itself manifestation or projection of quite different cell property namely the acid-base relationships of nucleic and cytoplasmic material.

In other fields of investigation such as the examination of sputum specimens for acid-fast bacteria, the pathological examination of liver tissue, and the morphology of platelets and red blood cells, the issues and methods are quite similar. With this background we are now prepared to discuss other parameters which can be used in automated cytology.

To begin with there are the techniques for rapid flow analysis of cells in suspension. The Coulter counters make use of the volume displacement of electrolyte to measure cell size and thus automate the red blood count, white blood count, and platelet count. This one-dimensional cell examination has certain flaws however and is unable to distinguish between leukocytes and nucleated red cells. It is still valuable tool in the clinical laboratory and the latest version, Model S, has met with wide acceptance.

Another extremely intriguing technique developed by Kamentsky and Melamed, is the rapid-flow spectrophotometric analyses of biological cells. Cells stained in suspension are made to flow through dow channel where multi-wave length absorption coefficients are measured. By looking at large cell populations as they flow through the channel, estimates may be made of the ensemble distribution of these absorption coefficients. By proper selection of the dyes the absorption may for example represent protein or DNA densities. Through these techniques rapid mass cellular screening procedures may be developed.

In the techniques that deal with the cell images research is presently proceeding in two directions. First, and most radical, of the techniques are those involved with mathematical transformations of images. For example, the Fourier transform of the image may be observed in the back focal plane of the microscope objective under near monochromatic illumination. Since the magnitude or intensity of the transform is invariant to translation of the image, this technique could be useful for rapid flow image analysis.

Finally we come to the techniques and parameters with which I am most familiar—namely the quantitative treatment of those parameters that have previously been dealt with only in qualitative way.

As previously mentioned, the availability of powerful low-cost computers, and high-quality electro-optical systems makes it possible to construct systems for the automated analysis of cell images. Further the parameters of the image previously mentioned—size, color shape, and texture may now be rigorously defined and dealt with in quantitative sense.

Color may be dealt with in a number of ways. The spectral distribution of energy at any point in the cell image may be broken up into disjoint regions yielding set of numbers which represent the sampled spectral waveform. The chromaticity coordinates of the spectral distribution may be calculated to yield the "visual color" of the cell image point. This last technique is one that I have had particular success with in characterizing the color of Wright' stained leukocytes.

Cell shapes provide an interesting source of problems in the general field of shape description and recognition. Although no definitive techniques have been developed for this problem, number of mechanisms have been suggested for dealing with the shapes of simply-connected closed contours. Measurements of perimeter squared to area ratio (P^2/A) Blum's medial axis transform, or counting the number of minima and maxima around the cell perimeter all represent possible descriptive mechanisms. In this area it has been my experience that most reasonably constructed ad hoc procedures work sufficiently well to be of use in shape quantification.

Finally word about pattern recognition. Pattern recognition is not parameter or set of parameters but rather tool for the manipulation of parameter sets for the purpose of decision making. Since we have, a priori, indicated our interest in using parameters or features of the cell image (rather than the whole image itself) this indicates that we are interested in that branch of pattern recognition concerned with statistical decision making. These statistical procedures are fairly well documented and thus their inclusion in any automated screening system is quite straight forward.

In conclusion it can be said that automated cytology offers methods by which the tedium, expense, and mistakes of present cell examinations can be reduced while freeing the technicians and researchers for more creative self-satisfying and exciting work.

DISCUSSION

DR. ADAMS

SELECTION AND COMPOUNDING OF PARAMETERS

It is frequently suggested, when cytophotometric and other cytophysical results are not as concise as desired, that some additional parameters be incorporated in the methodological complex. I am not entirely negative about this. As well as being nonredundant, each parameter in candidacy should be concise and concisely measurable. That is, it should be as much so, or more so, than the parameters already methodologically incorporated. If it is not, it might be well to disregard the parameter. If it is more so, some serious thought should be given to disregarding one or more of the previously-incorporated parameters.

There is another route. That is refinement of the initial parameters. Any parameter to be evaluated should be concise, and the technique of measuring it should be concise. For instance, DNA per cell, I think, can now be regarded as a concise parameter. The techniques for measuring this parameter can be as concise as time and expense will allow. The size of a cell is not so concise—it is variable, depending on how the cell is handled. Neither are many of the methods for assessing cell size concise. The presence of a cell in a radiation field or an electric field serves to distort the field in a way that may be related to the size or the mass of the cell. It is frequently implied and/or taken for granted that the "may be" of the last sentence is "darned well is." Nonetheless, it remains a most interesting question in most instances.

I don't mean at all to obstructively drag feet on the compounding of parameters, but I think the pendulum has been swinging towards abandon in this connection. Now we can make or buy devices that can rapidly handle all kinds of things on as many channels as we can imagine, and permutate them *ad infinitum*. I am just worried that, in our rush to saturate the appetites of these devices, we may tend to lose our common sense. It seems sometimes like a mad scramble to connect input devices with computers.

It is now understood that an excised mammalian cell is a plastic blob limited by membranes and composed primarily of water and otherwise composed mostly of protein and nucleic acids. It has been stimulating to assume that the amounts and proportions of proteins and nucleic acid may vary according to the functional state of the cell, and a number of rather convincing demonstrations that this is so have been offered. The above abundant components are easily demonstrable cytochemically if not by direct absorption spectrophotometry. Other cytologic constituents and affinities are more difficultly demonstrable. It is hopefully assumed that, if we can measure enough affinities, constituents, size parameters and other physical parameters, we can detect cancer. So far as I understand it, the most hopeful parameters for cancer detection are irregularities in per-cell DNA amount, and in the case of some epithelial cancers, changes in cell size and shape. It seems to me, judging by the way that things are going, that when additional parameters are proved valid, such proof may well be the result of automated investigations, where multitudes of parameters are simultaneously evaluated for validity. But I doubt if the devices that finally come into common use will be very complicated.

When a mammalian cell is stored, fixed, treated, stained, hydrolized, dehydrated, squeezed, dragged, sliced, irradiated, etc., a certain parametric richness is created. This can well serve to make a lot of activity for the machines, and, to be sure some good may come of it.

Now, with holograms new on the scene, and the validity of optical fourier transforms established as an experimental tool, there exists sufficient motivation for the examination of the possible role of the laser in cytology. The extent to which the transform of an image can contain more information than the image is quite limited, though transforms are excellent for visualizing periodicities which may be concealed in the specimen or its image. The laser is, indeed, an excellent source of stable, high-intensity spatially well-defined, monochromatic illumination, and quite cheap when the expense of obtaining these characteristics with conventional optical systems is considered. The as yet not-well-evaluated cytological possibilities of coherence are a bonus. But the conceptual juncture of a smeared cell, for instance, and a holographic presentation seems rather clouded, at least for the time being by the uncertainly high probability of artifactual elaboration.

MR. BUTLER

Concerning the paper *Objective Cell Image Description* (Bartels, Bahr and Wied) it appears that some data reduction techniques which are well known in the field of image processing have been overlooked. The point is that an undue degree of pessimism may possibly be projected as to the ability of moderately high-resolution systems to process cell images rapidly It is difficult to see how more adequate descriptions can be developed without the added degrees of freedom provided by processing systems with reasonably high resolution.

The authors correctly point out that the digitizing of image information can produce very large quantities of data which must be sharply reduced before any useful information can be extracted In our general paper on computer image processing[2] the data-reduction process is broken down into several sequential steps which become more model-dependent in later stages. *Data compression* methods which are essentially model-independent have been known for some time to communications engineers[3, 5] and should be generally used in image processing systems.

Consider a scanning system which measures 256 × 256 ~ 65 000 partial extinction values to 4 bit accuracy (7 bit precision may be pushing measurement technology but too far) The measurement of 10 cell images per second would then lead to data rate of 2,600,000 bits/sec However simple hardware techniques such as run-length or coordinate coding can achieve compression factors of ~ 50 reducing the data rate to manageable 50,000 bits/sec

The subject of descriptive or structural methods in pattern recognition is an interesting one but I do not personally feel that these methods are necessarily appropriate for cytological work It is difficult to see how to derive descriptions of this type in statistically stable way In other words, a descriptive analysis of an image in which noise is present is apt to be subject to large and unpredictable fluctuations of the inferred structure

Regarding the general question of optimum choices of descriptors for cell images, it seems that much more thought should be given to relating the descriptors to meaningful biological invariants Eaves[4] states

> "The ability to assign metric parameter to visual data, even in the absence of precise definition of the appropriate unit of information seems to presage the emancipation of certain areas of biomedical science from an exclusive dependence on subjective evaluation

This is very good statement of the matter and we all subscribe to it as working rule. On the other hand, it is surely to be hoped that this is only an interim situation, and that such metric parameters can eventually be related to quantities important to the organism

It is true that considerable work has been done on measurements of total content of DNA and other nuclear materials, as is evidenced by the papers in this Symposium. Such determinations apparently do have biological importance I also believe that the various measures of image texture also show promise of biological interest. It should be quite instructive to study the Weiner spectrum of the cell image or some other characterization of the distribution of features as a function of scale To turn to another subject, color analysis has long tradition in biological work, but the successful quantitation of color measurements appears to depend on significant advances in the difficult biochemistry of selective staining techniques The idea of staining can be extended in many directions for example, radioactive labeling with (say) tritium can be viewed in this light.

Kinematic studies are also amenable to image processing techniques and show considerable promise of being biologically significant. In this connection, we have recently reported on an attempt to attach a quantitative measure to the motility of living cells by working from time lapse photographs.

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DR. HALE

Most biologists are not familiar with the patois of computer and mathematical language. They will, thus find it difficult to interpret terms such as—

real world implementation of the image"
zero-memory source"
entropy of the value ensemble
deviation from equiprobable value distribution
point clouds"
eigenvectors
"Markov process

It is essential to explain in interdisciplinary plain language what these and other terms mean if the concepts presented in this paper are to be understood, let alone accepted.

I agree that objective novel descriptors of the form used in this paper must be used if data amenable to detailed analysis is to be obtained. There are, however difficulties, for example, the definition of texture must be expanded. This is dealt with in the paper but not enough emphasis has been put on explaining that texture might mean different forms of analysis or perhaps, comparisons of different forms, apart from its mathematical use to define value sequences.

It is important to explain the significance of these descriptors as *biologists are trained to believe* what they see and find difficulty in accepting analyses of patterns which they do not understand

As stated at the end of the paper these analyses might yield the information we want but are a long way from having the hardware that would produce the data required in the time available for practical diagnosis.

Mr McMaster

Many strategies involving diverse parameters have been proposed for automatic screening in cytology These range from biochemical parameters (e g measurements of enzyme activity) to physical parameters (e.g measurements of electrical conductivity and electron spin resonance) Most attention however has been focused on parameters associated either with flow systems, which measure the optical absorption, fluorescence and scattering properties of cells, or scanning systems which basically measure parameters associated with or derived from the morphology of cells (e.g shape texture)

In the particular field of screening for cervical cancer in which I am involved, manual measurements by Tolled *et al* Diamond (and this symposium) and Rummer have shown that there is a greater frequency of cervical cells with large nuclei on positive smears than on negative smears This fact is of considerable importance and could be the basis of a successful automatic screening system. However it cannot be used directly on its own. A system designed only to count objects in a prescribed size range would inevitably count white cell clumps and debris Even with improved sample preparation (involving clump dispersal techniques and mucus dissolving agents) and presentation (involving track systems) it seems unlikely that the quality of the sample will improve to such an extent that the single parameter of nuclear size could be used alone in an automatic screening system.

A recognition strategy involving additional parameters which distinguish between cervical cell nuclei and all other confusing material such as cell clumps and debris is therefore needed and it is this requirement which would seem to exclude flow systems from this particular field.

We are currently testing a recognition strategy which satisfies the above requirements. The system, which is not an automatic screening instrument but a general purpose test bed for recognition strategies, scans photographs of selected fields of view A field of view is photographed if it contains either cervical cells with large nuclei (greater than 12 μ) or material which might defeat the recognition strategy The fields of view are scanned by a flying spot film scanner and the parameters are extracted by a general purpose digital computer By scanning individual fields of view there is never any doubt as to why the strategy is succeeding There is a danger if a strategy is tested by screening large numbers of smears and the final classification of the smear is used as the criterion of success, that a correct classification is obtained for the wrong reason This happened during tests on the Cytoanalyser

The present strategy consists of scanning a field of view to detect all objects in a predetermined size range which satisfy loose constraints on their shape and density It is anticipated that in a practical screening system this part of the strategy could be performed in real time by using multiple delayed coincidence circuits.

Those objects which are detected during stage one are subjected to a more detailed analysis in which the shape and texture of the objects are measured and combined to classify the objects as either cervical cell nuclei or not cervical cell nuclei. In applying this strategy to large numbers of fields of view no objects which are not cervical cells have yet been found to defeat the strategy and the time is fast approaching when we can make the transition from an experimental test system to a prototype practical machine.

Drs Mizuno and Tukahara and Mr. Satoh

Various considerations have been made in the selection of parameters to be objective quantitative and informative, as the first step toward automated cytology What have been mostly considered so far as the parameters are density and size of the stained nuclei by the Feulgen reaction and the acridine orange staining on the nucleic acid. However our experiences tell us that electrophotometrically there are so many problems in the comparison of measured values, because in the Feulgen reaction the errors in staining are rather high, and the acridine orange staining presents an unsteady light source and attenuation of fluorescence Further in order to discriminate the malignant cells from the normal ones, we are inclined to consider the great risk of erroneous diagnoses if based on a few parameters

Recently the measurement of cellular parameters in the flow system was announced, but it seems to us to be inadequate, though we have no experience on this facet of automated cytology chiefly concerned with uterine cancer It is very difficult and troublesome to suspend the cell evenly in the liquid for this purpose

From the view point of automated cytology for use in general check-up for the public mainly in search of uterine cancers, we have taken up the specimens stained by the most commonly practiced Papanicolaou method and aimed at the automatic recognition of normal and malignant cells, taking as many parameters as possible out of these specimens and judging these parameters collectively. For these parameters, we are considering the area of cells and nuclei, N/C ratio, density of nuclei, characteristics of nucleic outline, distribution of irregular cells scattered throughout the smear and the likes, though not the introduction of new parameters. Further making full use of maximum absorption by the specific wavelengths based on the microspectrophotometrical data, these parameters are led into the computer through the ITV in the form of electronic and digital signals.

Drs. Mullaney and Steinkamp

The opening remarks on the four papers on this topic are concerned with parameters which can be measured either now or in the foreseeable future using flow systems or computer assisted scans of single cells mounted on slides or tapes. Comments on the relative merits and potential of these two approaches will be presented in the Discussion of the Prescreening section of this Symposium.

Van Dilla and Fulwyler Paper

Van Dilla and Fulwyler have limited their opening remarks to flow systems and their potential. Their paper is straightforward in its survey of the types of biophysical parameters which can be measured singly or multiply on single cells using flow systems and current technology. Optical parameters, fluorescence in particular, appear to be quite promising. Determination of the biological state of the cell associated with given parameter or set of parameters is necessary if this approach is to be applied to clinical prescreening.

Bartels et al. Paper

Unfortunately, the bulk of the paper by Bartels, Wied, and Bahr on computer-assisted single-cell scanning is written in the jargon peculiar to communications theory and computer image processing and, as such, is rather difficult for the nonspecialist. The significance of this work will be lost on the more general medical audience comprising this Symposium. Their approach, TICAS (Taxonomic Intra-Cellular Analytical System), appears to be potentially quite powerful for the long run. It has successfully differentiated between two cell lines which were morphologically indistinguishable to the human eye. The point of the project is to develop an automated "super pathologist" with discriminatory powers exceeding those of the expert human pathologist—an ambitious goal. The machine performs point-by-point two-dimensional optical density scan of cell, and these data are the inputs to a computer. These data can be processed to give cell area, total extinction at a given wavelength, average extinction, and mathematical descriptions such as the frequency histogram of extinction values along a given scan line, the frequency histogram of differences in extinction values along the same line, and other abstract mathematical expressions constructed from the data. Thus the cell description is given by some combination of mathematical expressions rather than by the more classical descriptors such as morphology, etc. A good summary of the possibilities of the TICAS approach may be obtained from the papers presented previously by this group.[1–4]

Schmidt-Weinmar Paper

Schmidt-Weinmar proposes an interesting idea, that of using coherent monochromatic laser light to obtain the scattering amplitude and phase in all directions on living cells (holography). Although this may be somewhat difficult to accomplish at high speed, significant changes in these scattering patterns would presumably be attributable to different biological states, and prescreening could be accomplished by noting the scattering differences among cells. Along these same lines, Wyatt[5] has proposed a technique, differential light scattering, for identification of microorganisms. Rather than solve the inverse scattering problem (i.e. conversion of light scattering information into structural details of the scattering particle), Wyatt suggests that light scattering pattern or "signature" be experimentally found for each type of cell and that differences in these "signatures" be used for identification. Initial experiments on bacteria using this method have shown that there are indeed detectable differences. It would be interesting to see results on various mutants to determine if subtle differences could be detected by this method.

General Discussion

It is obvious from this Symposium that the ideal parameter or set of parameters for differentiation of normal from abnormal has not yet been found. At present, differentiation is accomplished by human detection of morphological features, one or more of which may be indicative of abnormality. The data obtained by eye become the inputs to complex decision-making machine, the human brain. Prescreening approaches such as the CYTOANALYZER, CYDAC, TICAS, etc., attempt to mimic or surpass the human eye-brain combination in obtaining quantitative information on much the same parameters man senses. These machines, which require elaborate computer dealt with in the paper but not enough emphasis has been put on explaining that texture might mean different forms of analysis or perhaps, comparisons of different forms, apart from its mathematical use to define value sequences.

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The flow system approach adopts an entirely different philosophy in requiring the machine to measure what it can best (namely physical or chemical characteristics which give rise to light scattering fluorescence or other optical or electrical signals) For fullest exploitation of this point of view one must measure promising cellular parameters and correlate these with the biological state of the cell population (i.e., do prescreening on machine terms rather than on human terms) The capability for high-speed measurement can be adapted from very sophisticated methods largely developed for nuclear physics. The problems remaining are largely correlation of measured parameters with pathological or nonpathological states of the cells and the search for new methods of making cellular features definitive of cancer demonstrable to electronic sensing instrumentation.

Thus, it appears that a greater cooperation between cytopathologist those engaged in basic cancer research, and instrumentation specialists is needed to determine (1) what parameters are most definitive of an abnormal condition and (2) how to automate their measurement adequately

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Dr. Roth

Cytoscreening by Measuring Acridine Uptake

Our approach to automated cytology was in the direction of finding a parameter with suitable discriminating ability whose measurement would be sufficiently stoichiometric to permit a statistical analysis based upon interactions with populatio of cells. By establishing standardized procedur conditions the loss of a critical chemical agent fro the reaction mixture to the cellular target a could then be measured from solutions by rout photometric methods. Additionally it was antic pated that a statistical approach would achie higher sensitivity for cancer recognition th might be obtained by measurements of substance at concentrations to be found in single cells.

Since the problem of cancer of the oral cav has been our main concern, the procedure wou be necessarily limited by the numbers of cel readily harvested from the oral mucosa (of th order of 10^3 cells, in our experience) Low co performance by the community hospital's labor tory and acceptance by the patient (often, in th category of cancer a self-neglectful and negat type) were important requirements.

We selected the measurement of some reliabl index of the DNA content of a population of 50,00 cells as our best parameter Acriflavine seemed t offer the most appropriate properties. We foun that, under stringent limiting conditions, th diaminoacridine dye binds with near-stoichiometr and high selectivity to native DNA in cells, ap parently by a preferential association with thymin sequences [1 2] The restricted concentrations of th dye which must be employed to prevent it fro binding indiscriminately to various anions othe than DNA, required fluorometric analysis, wit its gain of up to 1000 × the sensitivity of absorp tion methods. The high fluorescence coefficient o acriflavine proved advantageous. While the bindin of small cations to large polyanions is only in completely understood, it is widely believed tha acridines gain their strong binding affinity fo DNA from a cooperative set of interations whic include covalent bonds between ring nitrogens o the dyes and phosphates of the DNA backbone second-order hydrogen bonds between the diamino groups of the dyes and ring nitrogens of nucleotide bases, and a unique steric fit of the flat dye molecules between sequential base-pairs in the rigid DNA molecule To exploit these characteristics the following procedure was developed.

Oral squamous cells are obtained by swabbing or rinsing, and collected into normal saline solution No specific site is selected, the buccal mucosa serving best for the purpose The cells are not fixed, and storage at 4°C is recommended if they are to stand for longer than one day One advantage so gained lies in the fact that leukocytes rapidly autolyze when unfixed, and their DNA is then washed away in the following steps, thus eliminating a source of nonspecific increase in DNA content. The cell suspensions are washed twice in phosphate buffered saline solution, pH

5 0 They are then evenly resuspended, counted, and drawn up in populations of 50,000. These are then spun down to dryness and stained with 0 055 μg of freshly diluted acriflavine in the dark at standard temperatures with no more than 5 range of variation (a heating block at 35°C is best) After 10 min of staining with frequent stirring the cells are removed by centrifugation, and the clear supernate is measured fluorometrically A commercial filter fluorometer fitted with high pressure mercury lamp is suitable. We use Corning glass filters, CS 5–74 and CS 4–105 for primary and emitted light beams A spectrofluorometer is not advised, due to its low output of exciting light. At these concentrations acriflavine concentration is proportional to fluorescence and instrument calibration is therefore simple. Thus the essential steps comprise centrifugation, cell counting, dilution, and fluorometry all of which are readily automated. The modular construction of the Technicon AutoAnalyzer is well adapted to this task[3]

We are finding significant differences in dye uptake between normal cells and the apparently normal cells from the buccal mucosa of patients with squamous cancer of the oral cavity or nasopharynx[4-6] Results are not as good in cases of laryngeal cancer Currently an extensive field trial being conducted under the auspices of the USPHS to evaluate the method and to learn causes of error At this point, about 95 per cent of cancer cases and 5 per cent of "normals" show an abnormal level of dye uptake Interestingly the cancer cases give positive results whether or not they have been cured or treated corollary-wise, cells taken from apparently normal sites in cases of cancer take up about the same amount of dye as cells scraped directly from tumor These observations suggest an abnormality of DNA metabolism as precursor to the ultimate development of cancer and lead us to hope that we may find the method useful for predicting future carcinogenesis in predisposed subjects If this proves to be true, the appropriate place for the procedure may be in multiphasic health screening programs of the kind now under active development In this connection, we have found an increased dye uptake in smokers, in proportion to their cigarette consumption but as yet no correlation with alcohol intake has emerged

Statistical increases in DNA-related dye-binding can be attributed to such events as polyploidy polyteny enhanced DNA synthesis, prolongation of S or M phases in the cell cycle, and to structural changes such as methylation or uncoiling of DNA Thus, no single explanation for changes in dye-binding is possible Other nonspecific factors causing increased dye uptake include irradiation, infections with DNA-viruses such as herpes, and some conditions such as lichen planus. No doubt other spurious factors will emerge However if these do not become logistically unmanageable the method may have a secondary value in bringing to light conditions other than cancer which require treatment. We expect that the system will be applicable to screening for cervical cancer but since it requires cells with relatively intact DNA, it may not be useful for fragile, autolytic cells such as gastric epithelium Large amounts of intracellular mucin will probably negate the method, as will pleocytic populations such as occur in effusates.

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Dr. Sandritter

Paper by Bartels, et al

It would be helpful to readers if one would elaborate on "The model of Markov process Perhaps the authors would care to do so, or to indicate where in the text they have already done this.

The concept of sub programming to simplify the eventual classification seems to be key to pattern recognition by high resolution methods Do they also apply to low resolution devices?

Paper by Van Dilla and Fulwyler

The use of biologic internal standard is chancy business Changes from one animal to the next as well as possible deterioration of cells in preparation or in storage make such standards less creditable than commercially available one that can be prepared in large batches.

Such standard has been under study for some time and seems rather helpful because it can be manipulated in a number of ways.

I refer to the use of fluorochrome stained or unstained ion exchange *spheres. Most of these are* manufactured of polystyrene *and are cross linked* with divinyl benzene (DVB) It has been shown that the fluorescence of well washed, unstained spheres is directly proportional to the percentage of DVB in the *resin and to the total volume of the* individual spheres. Depending on the type of resin used, either red or green fluorescent spheres of constant fluorescent intensity can be produced with acridine orange. As well, it has been shown that the fluorescence *intensity is proportional to* the concentration of fluorochrome in spheres in the useful range of total fluorescence yield

With reference to the blocking of interfering fluorescence with proteins, it is useful to know that acridine orange follows the rules about complex formation and dissociation and the critical concentration of blocking agents which were first used effectively by John Scott. It has been possible to measure, and qualitatively distinguish heparins with single mast cells by this means.

DR. THORELL

Present day cytologic and histologic diagnostic decision-making" is based upon a vast empirical knowledge collected during more than 100 years. Before the construction of a usable microscope by Amici, Frauenhofer and others around 1824 and the development of various staining techniques, it was not possible to discriminate between a normal cell and a cancer cell. We are in a somewhat similar situation today when we are looking for parameter-descriptors suitable for a machine to distinguish between normal and pathological tissue elements.

Scanning of the conventional microscopic preparation *followed by an objective cell image* description" by various morphological and cytochemical parameters is valuable for gaining new basic knowledge about the nature of malignancy For practical diagnostic purpose, however it seems *scarcely useful* at all. Even with the most *rapid* microscopic scanning techniques, sufficient data from, say 5000 cells per specimen would take several hours to collect. Expensive computation work is also necessary to reach a final decision On the other hand, such work might be very useful to explore the significance of various parameters when designing flow system instruments which can pick up total *amounts per cell" cell volumes,* cell shapes etc. Another interest lies in the fact that computer processing of the microscopic cell image qualities including statistical analysis and computer decision-making might teach us somewhat *about* our own work as diagnostic cytopathologists.

In the flow systems in which absorption, fluorescence, scatter etc. can be recorded *with adequate* speed from a sufficient number of cells, the pattern obtained by different combinations of a *number of parameters cannot build up a classical* morphological cell image only rather abstract figures are displayed numerically or on the oscilloscope etc. In most cases it is not possible to *predict how and what such a display can tell us* in terms of discriminative properties. According to my rather limited experience, only rough guesses have now and then paid off The judgement about usefulness of parameters, alone or in different *combinations, therefore, must rest on pure em*pirical results from trials on well-defined clinical material Only from such work can it be hoped that machine-made discrimination between normal and pathological tissues and cells might find *some practical applications and so relieve the* cytopathologist of one or perhaps a few of his undertakings.

DR TYLKO

There are four papers offered for discussion on the topic of other parameters, descriptors to be considered. One is concerned with coherent optical *probing one with flow systems one with the analy*sis of colors and the fourth with the analysis of two-dimensional arrays of extinction values.

Schmidt Weinmar extols the virtues of visible laser light and discusses its theoretical use as applied to cells. He points out that by using a laser it is possible to measure the movement of living cells and claims that it would be possible to provide a more complete description of a cell than by using conventional optical methods. However at the present time the problem of recognition of cells *appears to be merely shifted to an analysis* of a photographically recorded interference intensity pattern

Van Dilla discusses a number of single parameters measurable in flow systems He then states that to distinguish several categories of cells it is necessary to measure several cell properties but does not provide any theoretical framework to allow the selection of diagnostic parameters He concludes Hopefully some combination of measured properties could be obtained which *would uniquely* define malignant cells, permitting automatic identification."

Young mentions flow systems and then briefly describes his work on calculating the total color of cell image points using chromaticity coordinates of the spectral distribution

Bartels briefly *describes the principals of zero and low resolution systems and then passes to high* resolution systems namely the analysis of a two-dimensional array of extinction values and suggests that such a system is not suitable *for large* through-puts. He discusses various values that can be extracted from two-dimensional arrays including image texture and granularity and also discusses the problems of sequential dependency and of data reduction. He contrasts the present geometric approach with necessity for a structural approach for analyzing highly complex patterns such as tissue sections. It is difficult to discern the central thread around which the various methods indicated might coalesce to form a practical clinical automated cytology diagnostic machine.

Though it is acknowledged that the field of cytology is still partly unknown, it is felt that a number of other basic concepts must be pursued. Efforts should be made to establish a general classification of all the forms of cells that can be encountered in biologic preparations. Further the various parameters measured and suggested for measurement should be related to an invariant. More stress should be placed on the cybernetic nature of the material under study. The dynamic nature of the material must be recognized as the material is derived from growing tissues which live in changeable environments.

Drs. Wheeless and Patten

Definition of Available Cellular Parameters with a Slit Scan Optical System

Attempts at automated cytology have been directed principally toward three levels of instrumentation. The first is an instrument which automatically measures one or two parameters of an individual cell or obtains a profile of a specific parameter on a large number of cells. The second level of sophistication is an automatic prescreening system. This requires an instrument with a high through-put rate that is capable of making a decision on individual cells as to whether they are normal or abnormal. However, such a system would lack the sophistication to classify an abnormal cell as to its specific pathologic state. At the third level of instrumentation, effort has been directed toward a highly sophisticated system capable of examining a single cell in great detail and performing complex calculations utilizing the informational content of that cell image to specifically locate that cell in a multi-dimensional parameter space.

Most attempts towards automated cytology to date have centered around zero or high resolution optical systems. A zero resolution system is one in which the limiting aperture (either field or measuring) is larger than the cell of interest. A high resolution system incorporates a very small field or measuring aperture and the cell of interest is, in effect, scanned in some manner by this aperture. These optical systems satisfy the (information) requirements for the first and last levels of auto-

In the following, another system type, particularly adapted to automatic prescreening is proposed. This system, a slit-scan system, provides both cytochemical and crude morphological data on the cell.

Zero Resolution System

In a zero resolution optical system, both the field and measuring apertures are larger than the cell of interest. As a cell passes through the measuring beam, a single parameter is recorded, e.g. absorption, light scatter or secondary fluorescence, at a particular wavelength. Multiple parameter measurements may be realized by utilizing several detectors and possibly several sources. Because zero resolution systems generate a single number per detector they have the advantage of being rapid and requiring minimal data processing. Zero resolution systems also eliminate focusing problems encountered in higher resolution systems.

Several static as well as flow-through zero resolution systems have been developed. A flow-through system developed by Kamentsky, Melamed and Derman is based upon cellular absorption at 253.7 nm which provides information on total cellular nucleic acid content. While such a system has the advantage of requiring no staining, it suffers from its inability to monitor the two nucleic acids independently. The measurement of other cytochemical parameters or a single nucleic acid requires staining.

Another class of zero resolution system utilizes fluorescence. Fluorescence based systems offer the advantage of increased sensitivity and no distributional errors as compared with absorption systems[3]. A fluorescence flow system by Van Dilla, Trujillo, Mullaney and Coulter[4] allows measurement of DNA-related secondary fluorescence of fluorochromed cells while a multi-parameter static cell system by Wheeless, Bahr, Wied and Patten[2] allows the measurement of secondary *fluorescence at a multiplicity of wavelengths on* selected cells. The use of a metachromatic fluorochrome such as acridine orange allows, under proper conditions, simultaneous measurement of several cellular substances through their fluorescence intensity at separate wavelength bands.

While zero resolution systems provide valuable information on specific *cellular substances* and parameter profiles on large populations *of* cells, they have failed to demonstrate a capability to provide sufficient cellular information to permit the decisions required for use as a pre-screening instrument. Until a stain is developed which will specifically label an abnormal cell, one must seek additional parameters for use in prescreening instrumentation.

High Resolution System

High resolution systems which incorporate a field or measuring aperture much smaller than a cell are used to scan single cells to provide a two-dimensional data matrix. The informational content of this data matrix is used to locate that cell in a multi-dimensional parameter space with the hope of eventually dividing that space into cellular classification subspaces. There have been several attempts at this type of analysis, the most recent and most sophisticated of which is the TICAS system developed by Wied, Bartels, Bahr and Oldfield[4] Such a high resolution system is ideally suited for detailed studies on individual cells but has inherent limitations when applied to other tasks. The number of bits of information obtained per cell would severely restrict its through-put capability and the automatic focusing problem for a high cell throughout rate would be formidable.

Slit-Scan Technique

A slit-scan technique provides a more complete set of cellular parameters than is possible with a zero resolution optical system without producing the large data matrix associated with a high resolution system. It represents a compromise on through-put and resolution.

The slit-scan method currently being studied by the authors records the secondary fluorescence of a cell as it passes under a slit aperture (Fig 6) The resulting graphic fluorescence contour shown as a continuum represents the averaged fluorescence along the cell. In practice this would be a series of discrete samples taken on the cell as it passes under the slit.

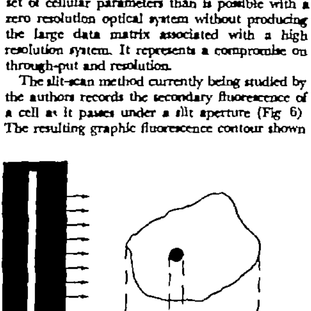

FIG. 6. Slit-Scan technique: Secondary fluorescence recorded as a cell passes under a slit aperture provides a graphic fluorescence contour

From the slit-scan contour recorded at 540 mm the fluorescence from the nucleus of a cell stained with acridine orange can easily be distinguished from any non-specific fluorescence of the cytoplasm. Integration of the area under the nuclear portion of the curve provides a measure of the DNA content of a cell. In a similar manner the RNA content of the cell is obtained from the fluorescence contour recorded at 655 mm. Because the boundaries of the nucleus and cytoplasm are recognizable from the set of fluorescence contours, the nuclear cytoplasmic ratio is also available. From these contours, rough estimates may also be obtained for relative nuclear area (nuclear size) and relative cytoplasmic area (cell size)

The recording of fluorescence slit-scan contours can be performed on cells in a static or flow through system. The additional parameters gained with this technique may provide the necessary data for a high throughput prescreening device.

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CLOSING REMARKS

DRS. BARTELS, BAHR AND WIED

We want to thank Mr Butler for calling our attention to model independent data reduction, and compression techniques. They are most valuable in pattern search situations, and since our own work so far has centered around visually selected individual cells they may not have received the attention that they deserve in the framework of this Symposium. But we can readily see applications in cell locating algorithms, and especially when pictorial elements are to be identified on the basis of morphologic features

The specifically mentioned run length, and coordinate coding techniques have their greatest efficiency for patterns with just two levels—black and white. We did employ variant of the run length method in the transmission of data to time-share system over long distance line, and it does offer valuable savings. Also, some work with predictive coding was carried out, in connection with certain covariance estimates in two dimensionally extended gray level arrays.

We have repeatedly emphasized that high spatial resolution is not absolutely essential for cell recognition purposes, but that moderate resolution is highly desirable. Because of this realization, we certainly did not try to consciously inject an undue element of pessimism into the discussion, although we are less optimistic than some of our fellow discussants about the cost efficiency.

Our experience has been that the required likelihoods for recognition of individual cells will demand moderate resolution of nuclear detail, and serious investigation of efficient special hardware components for rapid processing would be very worthwhile.

Four hundred image points per nucleus would be more than adequate, and 15 gray levels would be sufficient for most discrimination problems. Relatively high likelihoods can, however, be attained already by as few as seven to nine measurements per nucleus, provided high resolution measuring spot is employed.

Mr Butler seems to feel that four-bit precision is about the reachable limit of photometric readings. This corresponds to 6 per cent measuring error which would be entirely unacceptable, and is bad even for vidicon. We are not sure that there is not some misunderstanding. The Cytoscan distinguishes 180 gray levels, and easily has 7-bit accuracy with its highly stabilized tungsten source and built-in CAT. A matter of fact, in his article on computer analysis of photographic images, Mr Butler emphasized the 8 bit (256 gray levels) precision of the CYDAC scanner which for the application cited there is operated with CRT flying spot source.

Descriptive and structural methods which try to concatenate line-scan segments to reconstruct image primitives are indeed still quite removed from any practical application. I personally have, in fact, certain doubts whether or not the very approach of using formal syntax in this fashion is not computationally difficult to the point of being inefficient and not practical.

Such concept of structural approach however not the only one possible. In an attempt to obtain estimates for the required dimensionality of pattern space for morphologically distinguished objects, we have developed a system of computer programs which synthesize pictures. This has been used to generate pictures of tissue sections.

A sequential series of descriptive parameters and operators is used to generate two-dimensional patterns, and hierarchies of subpatterns. These operators and descriptive parameters are numerically encoded, their covariance matrices well known and there is no problem with statistical stability. The complementary program sections on pictorial element extraction are presently under development, but they use the same encoding methods. This system is descriptive, and structural in the sense that it uses a linear description to manipulate two-dimensionally extended information.

I do not agree with Mr Butler that the Wiener spectrum of a cell image reveals immediately apparent structure. Image texture to be sure implies periodicity in many instances, but not necessarily. In cell images we just do not seem to have periodically arranged structures.

Dr Schmidt Weinmar Paper

Diffraction pattern sampling is valuable in automatic photo-interpretation and in reconnaissance imagery. It is then possible to design sampling geometry which is centered, translation and size independent, and to obtain data reduction which is suitable to serve as sample signature for subsequent recognition processor. The decisive point here is, however, that in automatic photo-interpretation, one is primarily interested in detecting man-made structures, which distinguish themselves by regularities and periodicities, and to reject patterns indicating natural terrain. Cell images, both of normal as well as of tumor cells, produce diffraction patterns much as one obtains from natural terrain an ambiguous, irregular random pattern which does not appear much different for the two cell categories to be discriminated. This is not to say that there are no differences, but they would have to be extracted from the two-dimensional diffraction pattern by much the same textural analytic methods which can be applied to the original image.

The diffraction pattern is the spatial frequency representation of the cell, or to be more precise, since it is the intensity distribution of the diffraction pattern which is actually recorded, it is the power spectrum of the cell area transmission function. As rule there is simply not the kind of periodic regular structure in any of the cells under discussion that would let one take advantage of diffraction pattern sampling.

Dr Schmidt Weinmar is absolutely correct in pointing out the attractiveness of an optical preprocessing means, such as by matched complex filter. We are presently generating synthetic cell images which correspond to population averages of a very substantial number of cells of different cell types, and with granularities in the nuclei generated by the appropriate underlying two-dimensional stochastic process. Such computer-synthesized typical cell images could easily be perturbed, and Dr Schmidt Weinmar might be interested in testing some matched complex filters and their potential.

Rebuttal to Dr Saunders

To comply with Dr Saunders request A Markov process is a sequence of events which represent different states of a system. The sequence of values measured along the line scan in a scanning microscope is such a process. The gray value intervals into which each measured gray value falls represent the different states.

The state or gray value interval, into which a given value falls in a Markov process depends upon the preceding value for each pair of gray values, E_i, E_j, a probability is given that a value E_j occurs immediately after E_i occurred. The particular process described here is a finite Markov process

I am not quite certain how to interpret Dr Saunders next question. We have found the modular program design very flexible and useful and this applies also to employing different subroutines for the extraction of different features. Particularly in the self learning programs, one immediately obtains the information value of a particular feature and the average likelihood of correct classification by each feature.

In this sense I could foresee similar advantages for the modular design of software for low resolution devices in a research environment. In a production device, an established algorithm should best be implemented by whatever the most efficient way may be and without regard to niceties. I noticed, in reading the various contributions to the Symposium that rarely ever are linear combinations of parameters being discussed, and weighting procedures. As a matter of fact, error rates for individually measured parameters are often empirically determined assuming zero covariance between the parameter under consideration and other also measured parameters

Rebuttal to Dr Mullaney

I apologize to Dr Mullaney for the use of difficult language in my paper The field of automated image analysis in cytopathology spans several disciplines, from clinical gynecology at the directly applied end to rather complex mathematics. As active participants in an interdisciplinary field of research we all shall eventually have to learn each other s language

Simplification and the use of everyday language can be carried just so far before ambiguity sets in. Jargon comes into existence to preserve logical distinctions not made in everyday language. I wish to thank Dr Mullaney though, and appreciate his effort to give a more general presentation in his discussion remarks.

Dr. Schmidt Weinmar

Some recently published papers appreciably support the idea of a coherent optical determination of the structure of small semi-transparent particles, e.g., of living body cells. Wolf has worked out in detail the theory of a method for the determination of amplitude and phase of scattered light. He has also shown how these data, if recorded over a surface, allow the numerical reconstruction of the scattering object in three dimensions. His method has a maximum theoretical resolution power of about 10 times the wavelength of the light.

Carter[1] following the method of Wolf, has first obtained experimental results on model objects.

By computer simulation, Schmidt-Weinmar et al have studied another way to record magnitude and phase of the scattered light. The maximum theoretical resolution power with this method is about 1 wavelength of the light.

Coherent optical methods definitely seem to have a potential for successful application to cell recognition purposes. In particular the coherent optical structure analysis is one of the methods which should be considered if other parameters are to be measured. As several participants in this Symposium have pointed out, a cooperation between cytopathologist and engineer is highly desirable during the developmental phase of a new cytodiagnostic device.

At the present time, experimental difficulties arise in the recording of interference fringes by electro-optical image converters, due mainly to the low spatial resolution and rather limited gray level discrimination of these devices. Major industrial companies, however, seem to have strenghtened their research in order to overcome these limitations If one order of magnitude, both of spatial resolution power and of accuracy in measuring light intensity could be gained it would merely be a matter of optical technique and precision mechanics to build an actual coherent optical structure analyzer

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DRS VAN DILLA AND FULWYLER

Discussion by G H McMaster

Mr McMaster correctly points out that white cell clumps can be confused with abnormal epithelial cells by cervical cell screening instruments designed to measure cell or nuclear size. He goes on to say that additional parameters are needed to avoid this, which is certainly true, but we disagree with his conclusion that "It is this requirement which would seem to exclude flow systems from this particular field

Simultaneous measurement of DNA and volume in flow system should distinguish white cell clumps from epithelial cells in the following way Coulter volume spectrometry shows that the modal volume of epithelial cells from the buccal mucosa is about 40 times the modal volume of circulating leukocytes Microfluorometric measurements show the Feulgen-DNA content of both cell types to be the same, as expected This suggests the ratio of DNA content to volume as useful discrimination parameter If we take the leukocyte modal volume and DNA content as unity (units arbitrary) then the ratio of DNA content to volume for single leukocyte is $\frac{1}{1} = 1$ and the same is true for a clump of leukocytes For single epithelial cell, this ratio $= \frac{1}{40}$, very different number This contrast will diminish for polyploid epithelial cells, but even for octoploid epithelial cells the ratio is about $\frac{1}{10}$ and still very different from leukocyte clumps Hence, proper choice of cellular parameters and their arithmetic manipulation shows real promise of preventing confusion by white cell clumps and serves to illustrate the power of multi-parameter analysis

The light scatter signal available in flow systems may also assist It is possible that flat epithelial cells will produce more asymmetric scatter pattern than white cell clumps In addition sorting capability will allow these strategies of cellular discrimination to be evaluated and their effectiveness determined The paper Automated Cell Analysis An Integrated System (Opening Remarks) by Fulwyler and Van Dilla elsewhere in this Symposium discusses some of these points in more detail

Discussion by A M Saunders

We wish to point out that biological internal standard is the only possibility for the case we discussed in our opening remarks, namely measurement of absolute DNA content by the Feulgen procedure Only in this way can the entire system, including the staining procedure, be calibrated

W agree that standard fluorescent microspheres are ery useful in checking instrumental stability and performance and we have developed technique to produce very uniform polystyrene microspheres of selectable diameter and optical properties Fluorescent microspheres of about 10 μ diameter have been produced which give volume and fluorescence distributions with coefficients of variation of approximately 4 per cent in each case Use of these microspheres is discussed further in our 'Opening Remarks referred to above averages of a very substantial number of cells of different cell types, and with granularities in the nuclei generated by the appropriate underlying two-dimensional stochastic process. Such computer-synthesized typical cell images could easily be perturbed and Dr Schmidt Weinmar might be interested in testing some matched complex filters and their potential.

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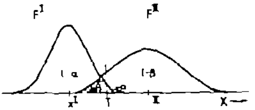

FIG. 1 The two distributions F^I and F^{II} overlap and correct, or incorrect classification decisions will occur with probabilities as follow from the setting of the threshold T

F and F are completely known, and follow distributions as shown schematically in Figure 1 For the time being let it also be assumed that equal prior probabilities exist for the occurrence of cells from both cell types When decision boundary T is set, on the X dimension, four possible outcomes exist for a decision

1) A cell of type I is correctly classified as type I The probability for such correct classification is 1 − α
2) A cell of type I is incorrectly classified as type II The probability for such an incorrect classification is that for an error of the first kind, i e of α
3) A cell of type II is incorrectly classified as type I The probability for such a decision is that of an error of the second kind, i equal to β
4) A cell of type II is correctly identified as type II The probability for such decision is equal to 1 − β and also called the "power of the decision rule

Error rates, and the power of the decision rule are thus completely determined by the parameters of the two underlying probability distributions F and F

A reduction in the permitted level of α automatically causes change in the power of the decision rule 1 − β The number of false alarms goes up, if type II were considered normal cell population for example and type I tumor cell For a wide range of values found in normal cells, the system ould hav to classify as "tumor cell One ay to achieve better discrimination is to change the definition of descriptor slightly so that the distribution functions F and F obtain different parameters, preferably greater means differences and or smaller ari nces This is often possible hen descriptors X based on textural image properties are employed

Another possibility is offered by a transgeneration of descriptors Sometimes tw image properties are observed none of hich leads by itself to ery good discrimination Combining the observed alue pairs, and forming ertain linear combination of th two, often l d to transgenerated singl and ver useful descriptor This is shown in Figure 2

Generally an proce lure which map th sample points from one cell type onto line d into an interval separate from the interval containing the transformed sample points of the other cell type is useful transgeneration

When cell are represent d by feat ure vector ith several componen s, as discussed further below transgenerat on frequently invol es mapping onto line ex end ng i the direction of grea est "between cell type dispersion This cor respond t the direc ion of the eigenvector of the between cell type ovariance matrix associated w th the greatest eigenval ie of the matrix

Returning to the one dimensional case In set ng the position of he decision boundary T one must consider the relative cost of the possible decisions Let th cost of making correct classification decisions be set to zero The relative cost of calling cell type I type II cell shall be set t unity An erroneous assignment of type II cell to type I shall then ha the relati cost of ω The cost factors can best be represented in cost, or loss matrix

$$\begin{array}{c|c|c|} & I & II \\ \hline I & c_{11} & c_{12} \\ \hline II & c_{21} & c_{22} \\ \hline \end{array} \qquad \begin{array}{c|c|c|} & I & II \\ \hline I & 0 & 1 \\ \hline II & \omega & 0 \\ \hline \end{array}$$

Also one will, n setting a decision boundary for which the expected cost is mo imized, want t consider the prior probabilities for the occurrence of cells from type I nd type II

The cost associated with classifying as I, given the examined cell is type I is

$$= p(I \mid I)p(I)$$

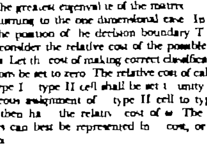

FIG. 2 Neither descriptor X_1 nor descriptor X_2 leads to satisfactory separation of the pattern points from samples I and II Forming new transgenerated descriptor Z, by linear combination of X_1 and X_2 leads to acceptable discrimination.

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Chapter IV Descriptor Definition and Measures of Discriminatory Power

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OPENING REMARKS

DRS. BARTELS, BAHR AND WIED

ASSESSMENT OF DESCRIPTOR EFFICACY

Human cytodiagnostic decisions are based upon a subjective assessment of a visual impression. It is qualitative evaluation of the cell image and remarkable for the almost instantaneous reduction of the pictorial information to a diagnostic decision. Automatic decision making is based on a quantitative and objective evaluation of the image information. In this, certain properties of the cell image are determined and expressed in the form of numerical values. The time required for the data reduction varies according to the complexity of the evaluation process, but it takes presently, in any case, longer by a factor of 100 to 10 000 than human visual assessment.

The determined "image properties" depend in their nature and definition greatly upon the underlying rationale of the recognition procedure.

The classical "geometric" approach is decision boundary oriented.[8-11] Image properties are defined so as to permit a mapping of the cell image into a certain region of a multidimensional decision space enclosed by a set of decision boundaries.

In the "structural" approach the cell image is described by the interrelationships found between sets of subpatterns nested in a hierarchic order.[4, 6-9] The "descriptors" involve the cell type specific mutual dependencies between these subpatterns and their levels, as defined and determined by a set of concatenation rules. The structural approach operates in an "open" decision space, open in the sense that the approach permits a description of new patterns or cell types, and recognition by the procedure.

In both cases, decisions are made whether assessment of a cell image property indicates membership in a certain class of cells or not. These are made according to classical statistical, and decision-theoretic principles. It should be pointed out that one is actually dealing with three levels of decision making:

1) Decisions concerning the information gained from a single image property.
2) Decisions based upon evaluation of a set of image properties, for an individual cell.
3) And finally, decisions concerning a whole set of observed cells from a clinical sample.

This hierarchic order of decision-making in itself illustrates the principle of the structural approach used in arriving at a quantitative definition for an inhomogeneous, structured pattern, or item—here a clinical cell sample on a slide. The various cell image properties form the primitives, combined into certain subpatterns in each cell. The next level considers the entire set of such subpatterns, and combines it into a feature vector. The sequence finally arrives at the cell sample pattern for the whole slide, on the basis of which a clinical decision is made.

The following discussion concerns primarily the discrimination as achieved by a single descriptor.

The classification problem can be seen as one where, for a certain variable X, a value of x is observed. X is distributed according to a probability distribution F^{I} for cell type I, and F^{II} for cell type II. The problem is to determine whether x came from F^{I} or F^{II}. Let it be assumed that both

The assumption of completely known probability distributions for F and F^{II} is not unrealistic in cell recognition work, where one can collect data from sizeable training sets Given new preparation one may employ preprocessing routines which would transform all measured raw data so as to conform the assumed probability distribution on which the setting of T is based

As rule, observation of just one variable rarely permits classification decisions with acceptably low error rates Instead, cell image is evaluated with respect to several image properties The numerical values found for these are combined to form the components of feature vector

The cell is then represented as a point in an observation space of as many dimensions as there are components in the feature vector The random variable X now is vector random variable distributed over p-dimensional space, with p denoting the number of its vector components F and F^{II} are probability distributions in this p-dimensional space The p several components of feature vector are as a rule correlated A compound decision that an observed vector random variable x came from the p variate probability distribution F or F must, therefore, take this covariance into account The estimate for probability density ψ_{I} or ψ_{II} as required for computation of the maximum likelihood ratio involves inversion of the covariance matrices Σ and Σ^{II}

A maximum likelihood ratio involving several feature vector components is easier to obtain when the several components are stochastically independent, and have separable probability distributions This can be accomplished by using as feature vector components, not the originally measured quantities, but rather components formed from linear combinations of these The new basis is found by a linear transformation of the covariance matrix The linear transformation represents the feature vector with component values following from the changed basis, and with mutually independent probabilities

The total probability of the joint occurrence of set of numerical values for the components of the transformed feature vector can then be computed simply as the product of their individual probabilities The problem eventually involves finding the eigenvectors of the covariance matrix

With the probability distributions of the different feature vector components now separable a maximum likelihood classification decision can be computed A method not infrequently employed in the biological sciences proceeds as follows For set of p-dimensional vectors, which consists of two subsets representing the two cell types to be discriminated, first the total covariance matrix is found Then the covariance attributed to the within subset covariance is removed, and the "between subset" covariance matrix found The eigenvector associated with its largest eigenvalue represents that direction in the decision space which has the greatest distance between subset variance [2]

The p-dimensional decision problem can then be reduced to a one-dimensional distribution, by considering only the mapping of each feature vector onto this between subset eigenvector Decision procedures as outlined at the beginning are then employed, measuring the discriminating power of the decision rule as outlined above

It is possible to attach an information measure to descriptor and to assess the efficiency of a descriptor in a data reduction scheme in terms of the number of bits which it carries It appears, though, that measure of discriminatory power based upon this faces several difficulties One is that one not dealing with complete system. Cell images show a continuous and wide variability in their texture, and distributions of textural measures There is always the probability that events with very low probability may occur Their consideration would naturally affect the entropy of the value ensemble The second difficulty is that an amount of information can only be attached in given system with known probability distribution The amounts of information carried by certain value group, used as descriptors, would therefore change with each discrimination problem A further difficulty arises from the strong sequential dependencies These demand that the *conditional* probabilities with which individual partial extinction values occur are considered when estimating their true information value

Such normalization difficulties could possibly find an answer by defining "bit fraction, i e the number of bits entering the descriptor expressed as fraction of the total number of bits recorded for the image

Even so there does not seem to exist convenient way in which the amount of information associated with descriptor can be related to the probabilities for an error of the first, and of the second kind

During the past few years, a growing literature is related to automatic cell recognition systems, to the procedures employed, and their critical evaluation The evaluation of each approach potential and progress directed towards development of the most promising procedure would be much helped if for each determined cell property the exact definition and data distribution parameters for the examined training sets were published, as well as pertinent data on the materials used in the training sets

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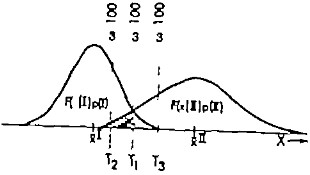

Fig 3 The effect of the relative cost factor ω on the setting of the threshold T is demonstrated

and the costs associated with any of the other three possible decisions are given by

$$c_{12}\ \ p(II \mid I)p(I)$$
$$c_{21}\ \ p(I \mid II)p(II)$$
$$c_{22}\ \ p(II \mid II)p(II)$$

The expected cost of the decision rule is the sum of these four components. A decision boundary is sought which minimizes this expected cost. Since c_{11} and c_{22} were set to zero, the expected cost E amounts to

$$E = c_{12}\ \ p(II \mid I)p(I) + c_{21}\ \ p(I \mid II)p(II)$$

or

$$p(II \mid I)p(I) + \omega\ \ p(I \mid II)p(II)$$

Figure 3 shows the two probability densities $F(x \mid I)p(I)$ and $F(x \mid II)p(II)$ and three decision boundaries positioned at T T and T The probability $p(II \mid I)p(I)$ is simply given by $\int_{-\infty}^{T} F(x \mid II)p(II)\,dx$, i.e. the horizontally shaded area in Figure 3 and $p(I \mid II)p(I)$ by $\int_{T}^{+\infty} F(x \mid I)$ $p(I)\,dx$, which represents the vertically shaded area. Minimization of the expected cost thus follows from

$$\frac{\delta E}{\delta x} = 0 = \frac{\delta F}{\delta x}\int_{-\infty}^{T} F(x \mid II)p(II)\,dx$$
$$+\ \omega \int_{T}^{+\infty} F(x \mid I)p(I)\,dx$$

or it places T at the point on the X axis where

$$F(x \mid II)p(II) - \omega F(x \mid I)p(I) = 0$$

which implies

$$F(x \mid II)p(II) = \omega F(x \mid I)p(I)$$

The likelihood ratio is therefore given by

$$\frac{F(x \mid I)}{F(x \mid II)} = \frac{p(II)}{p(I)\omega}$$

For any observed value x with a likelihood ratio larger than unity a classification decision $x \in I$, is made any x leading to a maximum likelihood ratio smaller than 1.000 would be classified as $x \in II$

The effect of the relative cost factor ω is to move the boundary from the point of intersection of the two probability distributions. An $\omega < 1.00$ moves the boundary towards smaller values of x, to T for example. $\omega = 1.00$ implies equal cost for decisions both ways, and leaves the boundary at the intersection an $\omega > 1.00$ moves it to higher values such as T_3 for example.

One may now plot this maximum likelihood ratio as a function of x and obtain a curve which lends itself as a direct indicator of the discriminatory power[8, 18] The steeper the gradient of the curve the more rapidly does the maximum likelihood ratio change as a function of x In practice, the log of the maximum likelihood is plotted to permit representation of the encountered wide ranges, and numerator and denominator are interchanged, so that maximum likelihood ratios >1.00 result on both sides of the point where the curve touches the abscissa (Fig 4)

Use of the gradients in the maximum likelihood curve, plotted as a function of x will permit a comparison of the discriminatory power between different descriptors only when all descriptors are expressed in comparable units. Curves of this type are therefore best plotted over a suitably scaled X dimension.

The discriminatory power as expressed by the gradient of the maximum likelihood ratio, and as associated with an observation x is, therefore, strongly dependent upon the distance $x - T$ or how far x is removed from the decision boundary When plotted as a function of this distance one obtains a curve which might be called the discriminatory power function

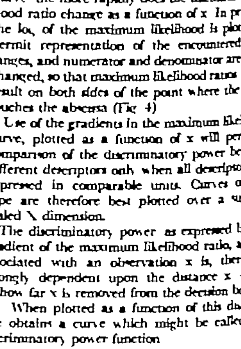

Fig 4 Plotting the maximum likelihood ratio or as shown here its log leads to curve the steepness of which indicates directly the discriminatory power of descriptor

The assumption of completely known probability distributions for F and F^{II} is not unrealistic in cell recognition work, where one can collect data from sizeable training sets Given a new preparation one may employ preprocessing routines which would transform all measured raw data so as to conform the assumed probability distribution on which the setting of T is based

As rule, observation of just one variable rarely permits classification decisions with acceptably low error rates Instead, cell image is evaluated with respect to several image properties The numerical values found for these are combined to form the components of a feature vector

The cell is then represented as a point in an observation space of as many dimensions as there are components in the feature vector The random variable X now is vector random variable, distributed over p-dimensional space, with p denoting the number of its vector components F and F^{II} are probability distributions in this p dimensional space The p several components of feature vector are as rule correlated A compound decision that an observed vector random variable came from the p variate probability distribution F or F^{II} must, therefore, take this covariance into account The estimate for a probability density ψ_I or ψ_{II} as required for computation of the maximum likelihood ratio involves inversion of the covariance matrices Σ and Σ

A maximum likelihood ratio involving several feature vector components is easier to obtain when the several components are stochastically independent, and has separable probability distributions This can be accomplished by using, as feature vector components, not the originally measured quantities, but rather components formed from linear combinations of these The new basis is found by linear transformation of the covariance matrix The linear transformation represents the feature vector with component values following from the changed basis, and with mutually independent probabilities

The total probability of the joint occurrence of set of numerical values for the components of the transformed feature vector can then be computed simply as the product of their individual probabilities The problem essentially involves finding the eigenvectors of the covariance matrix

With the probability distributions of the different feature vector component now separable, maximum likelihood classification decision can be computed A method not infrequently employed in the biological sciences proceeds as follow For set of p-dimensional vectors, which consists of tw subsets representing the tw cell types to be discriminated, first the total covariance matrix is found Then the covariance attributed to the within subset covariance is removed, and the "between subset covariance matrix found The eigenvector associated with its largest eigenvalue represents that direction in the decision space which has the greatest distance between subset variance [1]

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DISCUSSION

DR. HALE

Descriptor Definition

If a complex automatic cell screening apparatus is to be built then it is important that it must collect as much data as possible on multiple parameters of the specimen. These parameters need not relate to conventional cytologic subjective criteria correlating cellular appearance with malignancy They should seek to obtain any information which can be extracted from the specimen and correlated with the clinical characteristics of the patient from whom the specimen was obtained As the apparatus will be working at microscopic dimensions, it will only be able to utilize those methods of analysis which will offer sensitivity of detection of the minute signals presented (usually complicated by speed of detection) and of spatial resolution of the microscopic topography of the specimen

Analytical methods might include—

(a) Pattern recognition.
(b) Absorption analyses.
(c) Emission analysis
(d) Fluorescence analysis.
(e) Scattering detection.
(f) Polarization detection.
(g) Refractive analysis
(h) Reflective analysis.

The restrictions placed on the apparatus will limit the kinds of energies used to—

(a) *Visible photons.*
(b) Ultra-violet photons.
(c) Electrons
(d) Hard X rays.

Because of problems of instrument construction, the analyses will probably have to be done sequentially although there are ways of doing this semi-sequentially thus, for purposes of correlation, an overlapping sequence must be used—e g first step (a) and (b) second (b) and (c) etc.

If a large amount of data is to be collected, then it must be decided at an early stage what information is to be collected and how it is to be categorized. *Some of the problems* associated with such descriptor definition are—

(1) *Pattern Recognition*
    (a) Number of axes determined
    (b) Angular relation of axes
    (c) Continuous or discontinuous length of axes measurement.
    (d) Peripheral configuration
    (e) Within cell and between cell comparisons
    (f) *Cell population configurations* using the above criteria
(2) *Absorption Analysis*
    (a) Natural absorption
    (b) Induced absorption (staining) single or multiple
    (c) Total absorption of cell
    (d) Absorption of cellular organelles
    (e) Absorption distribution in cell
    (f) Spectral curves.
(3) *Emission Analysis* (includes fluorescence)
    (a) Natural emission
    (b) Induced emission (staining) single or multiple
    ( ) Total emission
    (d) Emission of cellular organelles
    (e) Emission distribution in the cell.
    (f) Emission spectra
    (g) Fading characteristics
    (h) Self absorption effects
(4) *Scattering Detection*
    (a) Amount
    (b) Orientation.
    ( ) Attenuation by "staining etc
(5) *Polarization*
    (a) Intensity

- (b) Plane
- (c) Intrinsic or extrinsic

(6) *Refraction*

- (a) Phase contrast image
- (b) Interference contrast image
- (c) Refractive matching techniques

If all of these techniques are to be used, then some relaxation of criteria of accuracy of measurement must be permitted in attempting to simplify constructional problems, e.g. correction of microscope optics for the different wavelengths used. Nevertheless, data from uncorrected lens systems could still be correlated with clinical criteria and may prove useful. Similarly between techniques some relaxation of criteria of accuracy must be permitted in the search for simplification. In addition to simplification of machine design, it is likely that specimen preparation, particularly that of a sequential nature, may also limit descriptor definition, e.g. if natural parameters are first measured, one must then decide how different stains (visible absorption or fluorescence) have to be applied and removed, if necessary, in the sequence. It is possible that there may be, between different stains, interactions which are in themselves descriptors.

If a wide variety of analytical techniques is used then it will be difficult in the first instance to decide the sensitivities used for each detection system and still work within a practical duration of analysis. It may be that the greater the number of techniques used, then the lesser the sensitivity of each is required, as there will be a greater probability of interaction. One, thus, will be balancing number of techniques, sensitivity and duration of each, and probability of interaction in choosing a system of analysis.

Descriptor definition must also be limited by the choice of data handling system. Here again one will have to balance choices of a relatively simple analytical system providing some sort of correlated interaction record which can be rapidly scanned for diagnostic features, with a complex analytical and data handling system. Perhaps one's priorities here will be settled by economic and personnel reasons rather than technical ones.

A systematic analysis of descriptor definition must be made based on:

(1) Analytical techniques qualified by selection of
  - (a) Sensitivity
  - (b) Speed of operation

(2) Preservation of specimen—
  - (a) Suspension flow techniques
  - (b) Discrete slides
  - (c) Transparent tapes with smear sequences

(3) Sequence of analytical techniques—this is dependent upon (2)

(4) Economic and personnel problems

Correlation with Clinical Diagnosis.

If the essence of an automated cytological screening and diagnostic system is to reach conclusions independently of classical criteria of cytopathological diagnosis, then the data obtained must be correlated with the clinical status of the patient. This means with diagnostic data other than cytological, follow up, and subsequent fate of the patient.

Diagnostic data should include:

(1) Complete history preferably as derived from an automatic history taking machine

(2) Physical characteristics based on a check list related to the diagnoses expected

(3) Other laboratory data—
  - (a) Routine clinical chemical data—check list
  - (b) Hormonal status-blood urinary steroid and, if possible, gonadotropin levels

(4) Therapeutic methods used

(5) Subsequent case history

As the criterion commonly used in deciding therapy is invariably, except for advanced cases, that of subjective cytopathological diagnosis, usually by cytology screening and then by biopsy, then the prognosis for the patient will be determined by those values with which we are not going to primarily relate the automated cytology data. Thus, at all times the automated data and the cytological subjective data must not be compared.

On the other hand, the probabilities of finding tumor cells in smears are so small that it would seem reasonable to teach the apparatus the nature of the normal and abnormal cells beforehand just by selecting them on conventional cytological criteria and presenting them to the apparatus. This should not include a selection of cytological criteria and when such cells are presented the apparatus must also analyze many other cells and cell groupings in the environment of the selected cells. In this way the apparatus can gain experience and may detect previously unknown population interactions in the smears. Once again, however, criteria detected in early instrument instructional runs should not mean that only those criteria are selected for further studies.

It is possible, however, that even this comparison of collected data with clinical diagnosis is already biased by the cytologic basis of most decisions to resect suspicious tissues. It is evident from the controversy over the nature of "carcinoma-in-situ" that we know little of how cytological changes progress in an untreated patient and, thus, we are biased in our decisions towards treating a disease that may not exist. By resecting a cervix we thereby remove the source from which a tumor might later have developed and, thus, we claim a cure for what is really a prophylaxis.

One may argue that there is no clinical data on patients subjected to automated screening as its justification is to detect cellular changes of predictive value before the patients present with frank disease. Nevertheless, clinical data must be adequately collected as we do not know what interactions are being sought.

It is also worth considering that prognosis might be improved by waiting until data collection has been obtained over a period of some weeks from different samples. Strengthening of interactions or appearance of new ones might be demonstrated.

CLOSING REMARKS

DRS. BARTELS, BAHR AND WIED

Dr Hale makes a very valid point in requesting that external information, such as data from the patient's history and other clinical laboratory data, be properly considered in assessing the patient's cell sample.

In a multiple iterative regression analysis we can obtain a precise estimate of each variable's contribution towards prediction of a criterion (normal or abnormal sample) This can be expressed, for example, by the acquired multiple regression coefficient of the variable linking it to the criterion to be predicted. It is quite conceivable now that in patients with widely varying sets of clinical data the predictive value of certain cell descriptors undergoes considerable change. Normally such between-patient differences of the variable covariance are pooled. By entering the external clinical information one could, however, have the automatic prescreener adjust its descriptor weights accordingly and thus make decisions more closely tailored to the particular patient's cell sample. At the present, we must admit that we do not have a feel for how much the covariance matrix of cell data is being deformed, for example by a pronounced change in hormonal levels in a patient.

Our own recent research in general cell recognition methods has time and again shown the value of various optimization strategies. They might well prove their value in finding, for each patient's sample that curvilinear discriminant through the multimodal, multivariate distribution of observed parameters, along which the test for the presence of abnormal cells should be conducted. We have recently developed a computationally feasible, highly efficient test statistic to make such decisions.

The external clinical information could, in a simpler way also be used to preselect test parameters for the computation of such a test statistic which must, in any case, form the basis for acceptance or rejection of the sample as normal.

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The Imprint Method for the Cytodiagnosis of Lymphadenopathies and of Tumors of the Head and Neck

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The importance of reliable preoperative diagnosis of tumors has been recognized for many years. Successful attempts to evolve methods for such preliminary diagnosis have been made since the beginning of this century. The frozen-section technique has long been used, as has the wet-film technique, in which scrapings from the tumor surface are taken for cytologic diagnosis. An analysis of more than 1,000 cases showed the wet-film method to be rapid and reliable when compared with histologic sectioning.

In the imprint technique, direct impressions are made of the surfaces of tumor sections. The literature contains a fairly large number of reports on the use of the imprint and wet-film methods in lymphadenopathies and mammary tumors. The cytologic diagnoses agreed well with the histologic findings in the same lesions. Saha and Laurdahu reported almost identical reliability in imprints and frozen sections of surgical specimens. Many writers have recommended that histologic studies should always be supplemented with cytologic imprints, since the latter permit more detailed assessment of the intracellular structure.

In order to make practical evaluation of the imprint method in preliminary diagnosis in the head and neck area and in mediastinal lymph nodes, we investigated 100 specimens obtained in department of otorhinolaryngology where tumor surgery is extensively done.

MATERIAL AND METHODS

The specimens

Biopsy specimens from 100 consecutive cases were examined. Fifty-nine of the specimens were grossly identifiable lymph nodes which had been excised mediastinoscopy according to Carlens' or from the head and neck area. Primary tumor of the oral cavity or the salivary glands was the preliminary diagnosis in 20 specimens and tumor metastases in lymph nodes in 21.

Imprint technique

The production of the imprints was simple. The biopsy specimen was cut through with sharp knife and the freshly cut surface was pressed against a glass slide. The slides were dried in air and rapidly stained by immersion for one and a half minutes in May-Grunwald solution and three minutes in Giemsa solution. A diagnostic report was then given to the surgeon. The average time from excision of the specimen to reporting was eight to nine minutes.

An additional imprint from each specimen was stained for 15 minutes in May-Grunwald solution and for 30 minutes in Giemsa solution.

Immediately after the imprints had been taken, the tissue specimens were fixed in formalin prior to the histologic examination.

RESULTS

The cytodiagnoses from the imprints are compared in Table I with the results of the subsequent histologic examinations. Two cases of tuberculoid lymphadenitis were cytologically misinterpreted as simple lymphadenitis. No malignant tumors were missed in scrutiny of the imprints and no histologically benign tumors were cytologically classified as malignant, i.e. there were no false positive or false negative tumor diagnoses. However, one adenocarcinoma was diagnosed from the imprints as epidermoid carcinoma and one epidermoid carcinoma was reported as anaplastic cancer.

All of the 16 tumors of the salivary glands, benign as well as malignant, were correctly diagnosed by the imprint method. As a rule, therefore, there was good agreement between cytodiagnosis from imprints and histodiagnosis from tissue specimens. The cell populations on the imprints corresponded well in most cases with those observed histologically. Figures 1–4 present some typical imprints and the histologic sections from which they were taken.

DISCUSSION

The percentage of error in cytodiagnosis from imprints was small in this study. The overall frequency of discrepancies between the cytologic and the histologic diagnoses was only 4 per cent. In no case was there misinterpretation of tumors

TABLE I *Comparisons Between Cytodiagnoses from Imprints and Histodiagnoses*

	Histologic diagnosis									
Cytologic diagnosis	Simple lymph-adenitis Anthracosis	Tuberculoid lymph-adenitis	Malignant lymphoma	Epidermoid carcinoma	Adenocarcinoma	Anaplastic cancer	Mixed salivary gland tumor	Papillary cystadenoma lymphomatosum	Oxyphil cell adenoma	Sarcoma
Simple lymph adenitis Anthracosis	45	2								
Tuberculoid lymphadenitis		8								
Malignant lymphoma			4							
Epidermoid carcinoma				12	1					
Adenocarcinoma					6					
Anaplastic cancer				1		8				
Mixed salivary gland tumor							9			
Papillary cystadenoma lymphomatosum								2		
Oxyphil cell adenoma									1	
Sarcoma										1

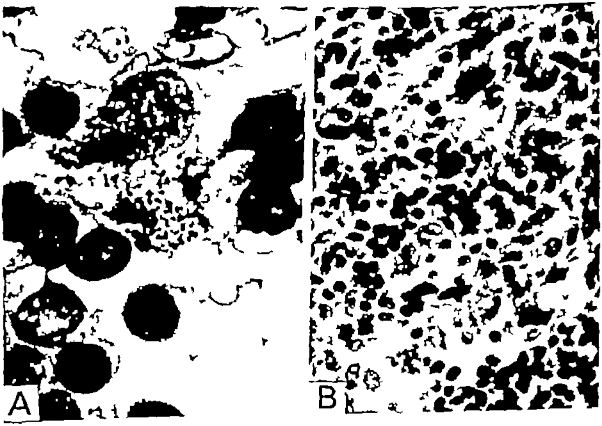

FIG. 1 *A* Cytologic imprint of an anthracotic lymph node with normal lymphoid cells and macrophages containing anthracotic pigment. May-Grunwald-Giemsa ×1200 *B* Section from the same gland, showing lymphocytes and pigmented macrophages van Gieson ×680

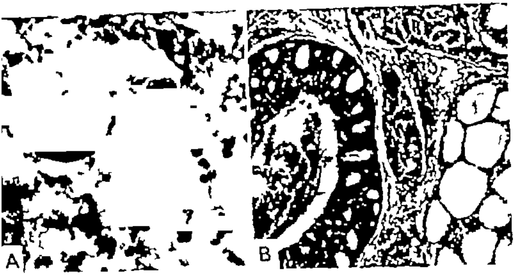

FIG. 2. *A*. Imprint from an adenoid cystic cancer with typical globules of mucus and discrete aggregations of epithelial cells. May-Grünwald-Giemsa ×550. *B*. Section from the same tumor showing mucus-filled spaces. Hematoxylin-eosin ×220

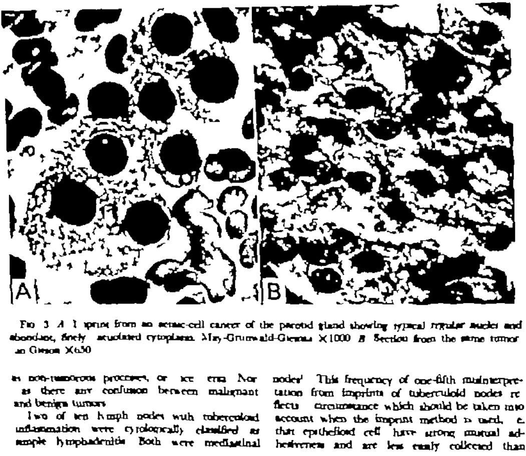

FIG. 3. *A*. Imprint from an acinic-cell cancer of the parotid gland showing typical regular nuclei and abundant, finely vacuolated cytoplasm. May-Grünwald-Giemsa ×1000. *B*. Section from the same tumor. van Gieson ×630

as non-tumorous processes, or vice versa. Nor was there any confusion between malignant and benign tumors.

Two of ten lymph nodes with tuberculoid inflammation were cytologically classified as simple lymphadenitis. Both were mediastinal nodes. This frequency of one-fifth misinterpretation from imprints of tuberculoid nodes reflects a circumstance which should be taken into account when the imprint method is used, i.e. that epithelioid cells have strong mutual adhesiveness and are less easily collected than

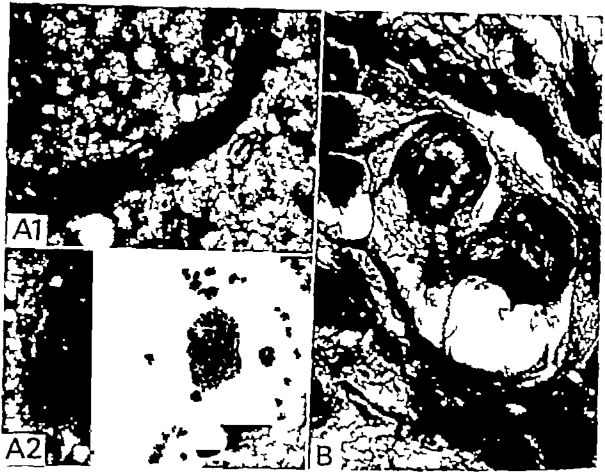

FIG 4 *A1* Typical fiber cells with cytoplasmic lamellae in an imprint from an epidermoid carcinoma. May-Grünwald-Giemsa ×1000. *A2* Multinucleated cell of malignant type with cytoplasmic lamellae in an imprint from epidermoid carcinoma. May-Grünwald-Giemsa ×1000. *B* Section from the same tumor The cytoplasmic lamellation is clearly visible in the central binuclear cell. Hematoxylin-eosin ×1000.

tumor cells on imprints. In the two mentioned cases, therefore, the epithelioid cells on the imprints were too scanty to permit a diagnosis of tuberculoid inflammation Imprint cytology thus seems to be less valuable for typing inflammatory processes than for distinguishing neoplastic from non-neoplastic lesions

Our observations agree well with other writers experience of imprint cytology in mammary tumors[8, 11, 14] The reliability of the imprint method observed in the present material agrees with that of frozen-section diagnosis[14] Though we have not made systematic comparisons between the frozen-section and the imprint technique, the latter appears to have some advantages It is more simple and requires less expensive equipment, and it is at least equally rapid. These qualities would seem to compensate any slight differences in diagnostic reliability though equality of reliability has been demonstrated[13]

In parotid-gland tumors, aspiration cytology has given increasing accuracy of diagnosis According to our limited experience, these tumors are suitable for the imprint method, which permits satisfactorily reliable classification of the tumor types

SUMMARY

The purpose of this report was to point the advantages of the imprint method, which is as yet relatively little known and used. We found the method to be well suited for the diagnosis of tumors in the head and neck area and of cervical and mediastinal lymphadenopathies, though somewhat less reliable in the differentiation of tuberculoid from nontuberculoid inflammation. The imprint method requires the services of an experienced clinical cytologist and may then replace the more complicated frozen-section method

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Morphology of Exfoliated Cells in Benign Gastric Ulcer

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Exfoliative cytology is a useful and accurate complementary diagnostic method in the investigation of gastric lesions.[2, 4-10, 17, 19, 21, 22] Of these lesions, gastric ulcer is the most frequent, and the differential diagnosis between malignant and benign types is clinically of utmost importance. Roentgenographic and endoscopic methods are the most important tools in this differential diagnosis but cytology plays an important role especially in those patients in whom the indirect methods are inconclusive or give conflicting diagnoses as to the nature of the gastric ulcer. The cytologic diagnosis also presents problems that should be recognized, in order that its accuracy be optimal. The two main problems of cytology are (a) the relatively high rate of false negative results in the presence of malignant ulcers[4, 21-23] and (b) the presence of atypical cells in the presence of benign gastric ulcers.[1, 3, 22, 23, 24]

To evaluate and further identify the atypical cells seen in benign gastric ulcers the present study was undertaken.

MATERIAL AND METHODS

A detailed review was done of all cytology smears in 524 cases of benign gastric ulcers, seen at the University of Chicago (Laboratory of Gastrointestinal Cytology) during the period of April 1955 to December 1969. As seen in Table I 476 patients were studied by the saline washing method[17, 21] and 48 patients by direct vision brushing method.[4] A follow-up of at least 12 months and extensive clinical studies determined the nonmalignant nature of the ulcers (in 127 cases by surgery and/or biopsy). A study of the clinical role of cytology in the differential diagnosis of these 524 patients and 68 other patients with malignant ulcer seen in the same period, is being published separately.[4]

RESULTS

Our review indicated several distinct degrees of cellular atypia in benign gastric ulcers, especially in the cases examined by the direct vision method in which cell preservation is optimal.

The least degree of cellular atypia, present in almost every instance of benign gastric ulcer is characterized by moderate nuclear enlargement, chromatin clumping and by the presence of one or more small round and regular nucleoli (Fig. 1). In some cases, especially with the saline washing method, a peculiar mixing of hyperchromatic and hypochromatic nuclei was seen. As this type of atypia was not observed in direct vision smears, this appears to be an artifact due to poor cell preservation.

A moderate degree of atypia is characterized by pronounced anisocytosis and anisonucleosis, some nuclear pleomorphism, and by the frequent finding of large irregular nucleoli (Fig. 2). The cells are always exfoliated in groups, with reasonably well preserved cell boundaries and cohesiveness. Goblet cells are frequently seen, and when their nuclei are atypical or pyknotic they should not be confused with signet-ring cells. This moderate degree of atypia was present in 50 per cent of patients.

Advanced atypia is characterized by marked dyskaryosis, nuclear pleomorphism, with irregular nuclear shapes, heavy chromatin clumping and large irregular nucleoli (Fig. 3). These cells occasionally are indistinguishable from malignant cells and accounted for the false positive cytology in the saline washing group. In three additional instances, they were correctly interpreted as benign atypical cells.

Even more advanced atypia was seen in two patients. It is characterized by the presence of isolated atypical cells. One of them (Fig. 4) closely resembled a signet ring cell, but the paucity of these cells was considered additional evidence of their benign nature because malignant cells always are seen in large numbers in direct vision cytology.

The two false positive reports in the direct vision material were issued because of the presence of

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Table 1. Exfoliative Cytology in Benign Gastric Ulcers
University of Chicago 1955–1969

Method	Benign gastric ulcers		
	Negative cytology	Positive cytology	No. of cases
Saline or Ringer washing	475	1	476
Direct vision brushing	46	2	48
Total	521	3	524

(Papanicolaou staining method)

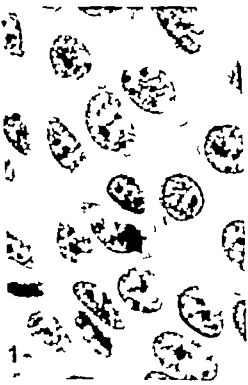

Fig. 1. Minimal degree of cellular atypia in benign gastric ulcer. There is moderate nuclear enlargement, chromatin clumping, and each nucleus has one or several small round nucleoli. Papanicolaou stain (× 50

large numbers of irregular appearing mitoses. As reported elsewhere, this occurred early in our experience with the method, and since then we have learned that mitoses are rather frequent in such material, and that the fixation can produce irregular patterns.

Discussion

That highly atypical cells are seen by the cytologist is not surprising. Histopathologists have long been describing highly atypical cells and glands in the margins of gastric ulcers. [illegible] Dible in 1925 noted such atypical regenerative hyperplastic changes in 32 per cent of 125 gastric ulcers, and Newcomb,[illegible] in 1932, described similar changes in 29 per cent of 161 gastric ulcers. In the experience of Gomori, the prepyloric antrum area is especially prone to this response, producing irregular glands and highly atypical cells difficult to distinguish from carcinoma. In the opinion of Wilensky and Thalhimer[illegible] these changes usually are hyperplastic and are not neoplastic. Some of these changes have been interpreted as evidence of malignant degeneration of previous benign gastric ulcer. However, these findings probably represent only atypical regenerative changes.

An entirely different situation is represented by the cases in which gastric carcinoma has undergone peptic digestion. Such malignant ulcers can

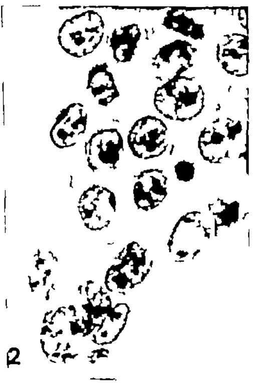

Fig. 2. Moderate degree of cellular atypia in benign gastric ulcer. There is anisocytosis and anisonucleosis, and each nucleus has one or two large irregular nucleoli. Papanicolaou stain (× 650)

heal only to break down again later[14] [14] Confirming the experience of Palmer and Humphreys[14] that carcinomas undergoing peptic digestion simulate the benign ulcer but still have diagnostic signs of carcinoma, Japanese workers have abundantly shown, as we have noted in a recent review[1] the usefulness and accuracy of the endoscopic classification of superficial ("early") gastric carcinoma in the vast majority of ulcerated type lesions.

The cellular atypias seen in the present study have received relatively scanty mention in the literature, except for the first or minimal degree of atypia that has been reported as "active cells" in patients with pernicious anemia, chronic gastritis, benign gastric ulcers, and accompanying the malignant cell in gastric carcinoma.[1] [14] [20] [22]

The moderate and advanced degrees of cellular atypias observed in our study are quite similar to the changes described in the "gastric-ulcer cell" of Witte and Bressel[23]

The use of intravital fluorescent staining with acridine derivatives by Witte[24] in 94 patients with benign gastric ulcers, revealed an intensive coarse and granular fluorescence, indistinguishable from the one produced in tumor cells in 13 per cent of the cases An attempt to measure nucleolar size in an effort to distinguish benign from malignant cells was unsuccessful because of the overlap between the measurements. In a similar study Vilardell[20] did not observe a nucleolar diameter in excess of

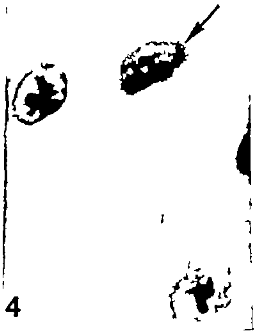

FIG. 4 Advanced degree of cellular atypia in benign gastric ulcer Presence of isolated cells, one of which is identical to typical signet-ring cell (arrow) Papanicolaou stain (× 1,250)

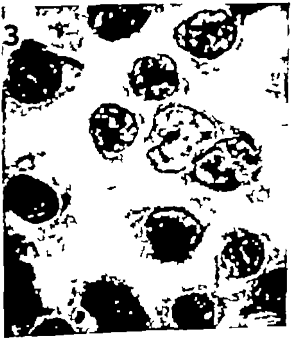

FIG. 3. Advanced degree of cellular atypia in benign gastric ulcer There is marked pleomorphism, especially of the cells seen in the center of the picture. Papanicolaou stain (× 650)

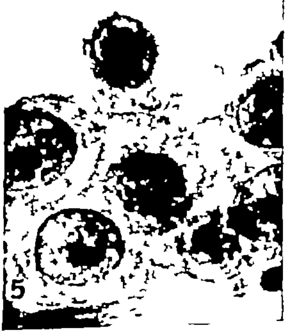

FIG 5. In this case of gastric adenocarcinoma, exfoliative cytology revealed cells with few morphologic criteria of malignancy The cells are seen in regular sheets their nuclei are regular in shape and chromatin content Papanicolaou stain (× 1,250)

4 micra in benign cells. In our experience, such large nucleoli occasionally occur in benign cells, and extremely large nucleoli, such as those measuring 5 micra or more, are seen only in a few carcinomas.

Miyake *et al* made a detailed study including phase-contrast and electron microscopy of 27 cases of gastric cytology in which a Class III of the Papanicolaou classification had been established. The final histologic diagnosis was benign lesion in 17 cases (3 adenomatous polyps and 14 benign gastric ulcers) and adenocarcinoma in 10 instances. The most striking difference was in the structure of the nucleoli. In all instances of adenocarcinoma they showed vacuolization (in 12 to 50% of the cells in each case), while the benign lesions demonstrated compact nucleoli (at least 97% of the cells in each case). This finding was easily demonstrated in phase-contrast microscopy.

The difficulties faced by the cytopathologist are further complicated by the occasional instance of gastric carcinoma in which all the cells have so little atypia (Fig. 5) that they could be mistaken for benign cells. However, careful screening of the material almost invariably will reveal some isolated cells, either typical signet-ring cells or their "foamy cytoplasm" variant. Richards and Spriggs, encountered the same difficulties, and stated that "in trying to diagnose carcinoma of the stomach from cytologic evidence we are working very near to the margin of what is humanly possible."

SUMMARY

A detailed review of all cytologic smears in 324 cases of benign gastric ulcers was made. The study revealed several distinct degrees of cellular atypia. The least degree, present in almost every instance, is characterized by moderate nuclear enlargement, chromatin clumping, and by the presence of one or more small round and regular nucleoli. A moderate degree of atypia is characterized by pronounced anisocytosis and anisonucleosis, some nuclear pleomorphism, and by the frequent finding of large irregular nucleoli. Advanced atypia is characterized by marked dyskaryosis, nuclear pleomorphism, with irregular nuclear shapes, heavy chromatin clumping, and large irregular nucleoli. These cells occasionally are indistinguishable from malignant cells and accounted for one false positive cytodiagnosis. The most advanced degree of atypia was seen in two patients, and was characterized by the presence of isolated atypical cells, closely resembling signet ring cells.

These atypias are important to recognize because of the potential diagnostic error. They correlate with atypical histologic changes seen in chronic peptic gastric ulcers.

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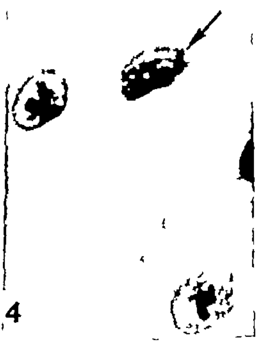
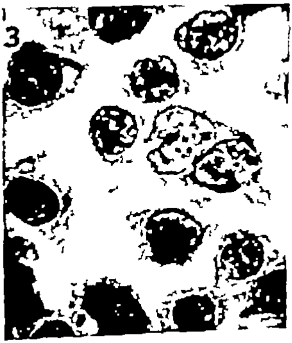


FIG. 4. Advanced degree of cellular atypia in benign gastric ulcer. Presence of isolated cells, one of which is identical to a typical signet-ring cell (arrow). Papanicolaou stain (× 1,250)



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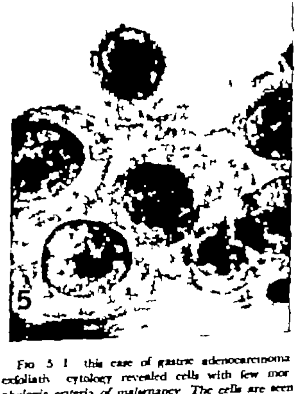

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These atypias are important to recognize because of the potential diagnostic error They correlate with atypical histologic changes seen in chronic peptic gastric ulcers

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The Cytologic Diagnosis of Tissue Repair in the Female Genital Tract

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The purpose of this paper is to describe cytomorphologic and cytochemical findings in cases of tissue repair in the female genital tract, and to demonstrate the rate of occurrence of these changes in routine cytologic specimens.

MATERIAL AND METHODS

The computerized cytology, histology and treatment file system of the University of Chicago, covering 150,959 patients was searched to identify cellular samples in which cytopathologists or cytotechnologists observed cells apparently deriving from "tissue repair." The code for this diagnosis was inserted on the file system since January 1969. In *this way only 45,000 patients* were included in this study.

The identified slides were re-examined, and results correlated with clinical findings and histories.

Supplementing this retrospective examination, comparative cytomorphologic and cytochemical study was carried out on samples from 50 patients who had recently undergone *hysterectomy*, cauterization of the cervix, or irradiation therapy or who had clinical evidence of severe cervicitis, while exhibiting cytologic evidence of "tissue repair." Cell samples were fixed and stained according to the Papanicolaou technique. Especially selected cases were fixed in 10 per cent buffered neutral formalin and stained with Masson trichrome method[1] for detection of possible elements deriving from the connective tissue.

The Papanicolaou stained sample was used to assess morphologic features (such as staining reaction, nuclear chromatin pattern, isolated cells, groups of cells, etc.). The slides were destained then restained by the Feulgen reaction for quantitative cytochemical assessment of DNA content. Absorption measurements were obtained on 630 cells at 565 nm wavelength by means of an on-line scanning microscope computer system. Some of the computer programs originally designed for pattern recognition[2] can be easily adapted to measurements of cellular and nuclear size and to the print-out of the total extinction. The programs used (TICAS 8, and TICAS 11) have been described elsewhere.

RESULTS

Analysis of the records from the computerized file *showed the occurrence of "tissue repair" as* seen in Table I.

In the Papanicolaou-stained material the cells believed to be derived from reparative tissue may lie isolated (25.3%) or arranged in groups (74.7%). The cells have varying degrees of cellular differentiation. The total number of cells per slide suggesting tissue repair varied considerably (212 ± 147). *More cells were found after recent* radiation therapy, followed by patients with severe cervicitis, patients after hysterectomy and by patients who were recently cauterized or biopsied.

In cells believed to be of epithelial origin by cytomorphologic criteria (Figs. 1 and 2), the cytoplasm was usually cyanophilic (63.2%), sometimes eosinophilic (29.1%) and less often indeterminate (7.7%). In 450 isolated cells suggestive of tissue repair the mean cell area was relatively small (173.1 ± 35.2 square micra).

A moderate degree of anisocytosis was present. The nuclei having a mean value of 27.2 ± 7.1 square micra had a relatively small degree of anisonucleosis. The chromatin pattern was classified as "uniformly finely granular" (59.7%), "finely granular with chromocenters" (38.4%) or "coarsely granular" (1.9%). They contained as a rule eosinophilic macronucleoli, which were single (73.5%) and multiple (26.5%). Most of the nucleoli were regular in configuration but some occasional irregular nucleoli were found. Mitotic figures were found in 4.3 per cent of the cases.

Dr. Bibbo is Assistant Professor, Department of Obstetrics and Gynecology; Miss Keebler is Chief Cytotechnologist and Educational Coordinator, Laboratory of Cytopathology; and Dr. Wied is Blum-Riese Professor of Obstetrics and Gynecology and Professor of Pathology.

The authors wish to thank Mrs. Mafalda Ochoa for her assistance in the cytochemical studies and measurements. The study was supported by a grant from the United States Public Health Service (1 R18 HS-00025-02) (G. L. W.).

TABLE I

Rates of Occurrence of Tissue Repair	
All patients (i.e. 45 000 cases)	1.04% (= 468 patients)
Patients after recent radiation therapy	3. 5%
Patients after hysterectomy (first post-surgical cell sample)	29.5%
Patients with severe cervicitis	21.1%
Other patients who were recently cauterized or biopsied, etc.	16.9%

Some groups of cells were observed whose origin were questioned. (Figs 3, 4, 5, 6) The cyanophilic cytoplasm (67.0%) and occasionally the eosinophilic cytoplasm (27.5%) is characterized by long, ribbon-like extensions (Figs. 3, 4 and 5) Between the network produced by these structures there may be relatively clear areas (Fig. 6) Aggregates of cells with interlacing strands of material were found in 23.4 per cent of the cell groups. Because the cytoplasm of these cells was poorly delineated, mean cell area could not be determined. Nuclei were found to have a mean area 24.3 ± 5.2 square micra. Their chromatin pattern was classified as uniformly finely granular in 42.3 per cent, and finely granular with chromocenters in 57.7 per cent.

Macronucleoli were observed in 77.9 per cent of these cells, being single in 68.4 per cent and multiple in 31.6 per cent. The nucleolar configuration was regular in the majority of cells but some occasional irregular nucleoli were found.

Cellular debris, polymorphonuclear leukocytes and erythrocytes were found in varying degrees of preservation in these samples. Histiocytes are also present in some instances (19.6%)

Material stained with Masson's trichrome method showed the following results. In cells believed to be of epithelial origin the cytoplasm was green in all cells, the nucleus gray and the macronucleoli red. In those cells of unknown origin, the cytoplasm and the ribbon-like extensions were green, the nucleus gray and the macronucleoli red. Worthy mention is the fact that most of the normal epithelial cells had red stained cytoplasm and dark red nucleus. Some intermediate cells, some parabasal cells, as well as some squamous metaplastic cells, had green cytoplasm and gray nuclei.

In regard to the Feulgen stained material, the DNA content values for 0 lymphocytes and 630 cells from tissue repair are shown in Figures 7 and 8. The lymphocytes showed a concentration of values around 1L, 80.3 per cent of the tissue repair cells showed a concentration of values around 1L, and 19.7 per cent around 2L values.

DISCUSSION

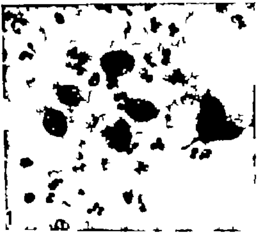
From the analysis of the computerized file it is shown that the overall rate of occurrence of tissue repair in the female genital tract in our material is 1.04 per cent. This is significantly different from the occurrence after recent radiation therapy (32.5%) after hysterectomy (29.5%) or in patients with severe cervicitis (21.4%) or in patients who were recently cauterized or biopsied (16.9%). From the data obtained, it seems more likely to find a larger number of cells suggestive of tissue repair in patients after recent radiation therapy or with severe cervicitis than in patients after hysterectomy or patients who were recently cauterized or biopsied.

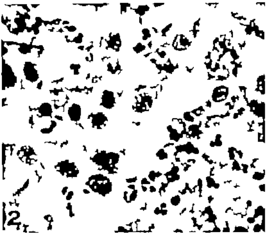
It is believed that the cells derived from reparative processes are epithelial cells with varying degrees of differentiation, the origin of which can be ascribed to either columnar or squamous epithelium.[1, 2, 3]

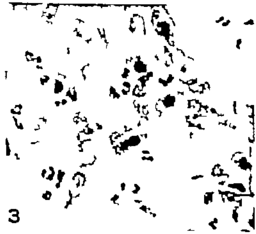
The cytometric measurements of these cells originating from the epithelium confirm the findings of Patten.[4]

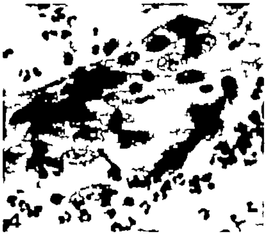
As previously mentioned, some unusual groups of cells in the Papanicolaou stained material were observed, whose origin were questioned. Due to the fact that they were in groups, had ribbon-like extensions from the cytoplasm forming a network, had uniformly finely granular chromatin pattern, and exhibited macronucleoli, it was thought they could have derived from the connective tissue. The resemblance of these structures with fibroblasts and collagen fibers is striking. Having this in mind, some specimens were stained with Masson's trichrome method.

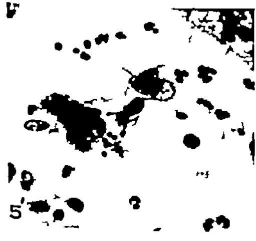
The results with this staining method were disappointing because it did not help to solve the problem of the origin of these cells. It was expected that only the elements derived from the connective tissue would stain green, as it occurs in histologic sections. However, it was noticed that all tissue repair cells stained green even the ones in which no doubt existed about their origin. In addition to that, some intermediate and parabasal cells, and some squamous metaplastic cells stained green also. The majority of epithelial cells stained red as expected. However the fact that some epithelial cells stained green, demonstrates the deficient specificity of this method for identification of connective tissue elements in exfoliated material. The Masson











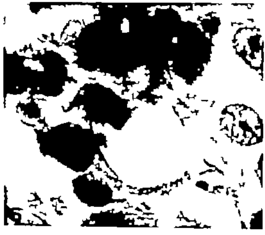


Fig. 1. Cells derived from reparative process of the uterine cervix in Papanicolaou stained material. The cells are probably of squamous origin and have prominent macronucleoli. × 606.

Fig. 2. Atypical cells, with conspicuous and often multiple nucleoli probably of glandular origin suggestive of tissue repair. Note abundant inflammatory background and red blood cells. Papanicolaou stain. × 606.

Fig. 3. Aggregate of cells with ribbonlike extensions from the cytoplasm forming a network resembling fibroblasts and collagen fibers. Masson trichrome stain. × 606.

Fig. 4. Specimen derived from reparative process. Note delicate chromatin pattern and prominent nucleoli. Papanicolaou stain. × 606.

Fig. 5. Group of cells originating in tissue repair. The elements could be derived from the connective tissue. Note size of cells as compared to normal squamous cells. Papanicolaou stain. × 606.

Fig. 6. Cell aggregate of apparent connective tissue origin. Note relatively clear areas between the networks of thread-like structures. Papanicolaou stain. × 1213.

TABLE I

Rates of Occurrence of Tissue Repair	
All patients (i.e. 45 000 cases)	1.04% (= 468 patients)
Patients after recent radiation therapy	32.5%
Patients after hysterectomy (first post-surgical cell sample)	29.5%
Patients with severe cervicitis	21.1%
Other patients who were recently cauterized or biopsied etc.	16.9%

Some groups of cells were observed whose origin were questioned (Figs. 3, 4, 5, 6). The cyanophilic cytoplasm (67.0%) and occasionally the eosinophilic cytoplasm (27.5%) is characterized by long ribbon-like extensions (Figs. 3, 4 and 5). Between the network produced by these structures there may be relatively clear areas (Fig. 6). Aggregates of cells with interlacing strands of material were found in 23.4 per cent of the cell groups. Because the cytoplasm of these cells was poorly delineated mean cell area could not be determined. Nuclei were found to have a mean area 24.3 ± 5.2 square micra. Their chromatin pattern was classified as uniformly finely granular in 42.3 per cent, and finely granular with chromocenters in 57.7 per cent.

Macronucleoli were observed in 77.9 per cent of these cells, being single in 68.4 per cent and multiple in 31.6 per cent. The nucleolar configuration was regular in the majority of cells but some occasional irregular nucleoli were found.

Cellular debris, polymorphonuclear leukocytes and erythrocytes were found in varying degrees of preservation in these samples. Histiocytes are also present in some instances (19.6%).

Material stained with Masson's trichrome method showed the following results. In cells believed to be of epithelial origin the cytoplasm was green in all cells, the nucleus gray and the macronucleoli red. In those cells of unknown origin, the cytoplasm and the ribbon-like extensions were green, the nucleus gray and the macronucleoli red. Worthy mention is the fact that most of the normal epithelial cells had red stained cytoplasm and dark red nucleus. Some intermediate cells, some parabasal cells, as well as some squamous metaplastic cells, had green cytoplasm and gray nuclei.

In regard to the Feulgen stained material, the DNA content values for 70 lymphocytes and 630 cells from tissue repair are shown in Figures 7 and 8. The lymphocytes showed a concentration of values around 1L, 80.3 per cent of the tissue repair cells showed a concentration of values around 1L, and 19.7 per cent around 2L values.

Discussion

From the analysis of the computerized file it is shown that the overall rate of occurrence of tissue repair in the female genital tract in our material is 1.04 per cent. This is significantly different from the occurrence after recent radiation therapy (32.5%) after hysterectomy (29.5%) or in patients with severe cervicitis (21.1%) or in patients who were recently cauterized or biopsied (16.9%). From the data obtained, it seems more likely to find a larger number of cells suggestive of tissue repair in patients after recent radiation therapy or with severe cervicitis than in patients after hysterectomy or patients who were recently cauterized or biopsied.

It is believed that the cells derived from reparative processes are epithelial cells with varying degrees of differentiation, the origin of which can be ascribed to either columnar or squamous epithelium.[2, 4, 5]

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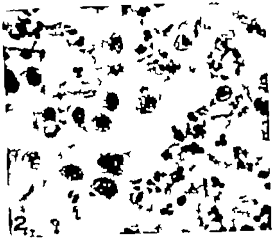

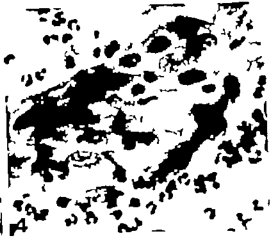

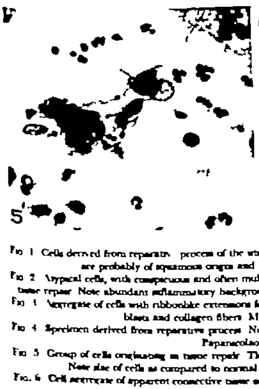

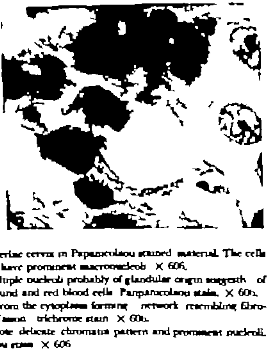

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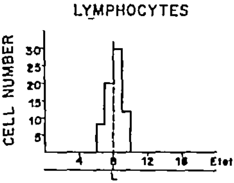

Fig. 7. Frequency distribution of DNA content in Feulgen-stained human lymphocytes measured for standardization of cells from tissue repair

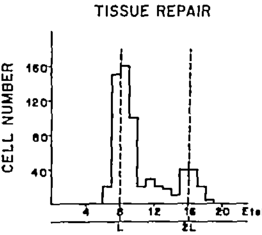

Fig. 8. Frequency distribution of DNA content in cells of tissue repair with concentration of values around 1L with a spread of a small number of cells with values ranging around L.

trichrome method in spite of being accepted in histopathology as specific for collagen, does not seem useful as such in exfoliated material. The original method suggested by Shorr[6] for staining of vaginal smears was a modification of the Masson trichrome variant of Mallory's aniline blue collagen stain. In the admixture of stains, the light green acted as a counterstain for nonkeratinized cells. In this way the less mature epithelial cells stain green, as observed in the present study.

In spite of these inconclusive cytochemical results, the cytomorphologic resemblance with fibroblasts and collagen fibers of the cells in question is so close that there is at least suggestive evidence that they derive from the connective tissue.

Photometric measurements of the relative DNA content in cells of tissue repair revealed some cells with values larger than 2L. Such values reflect DNA synthesis during cell proliferation.[7] Since 80.3 per cent of the measurements occur around the diploid and about 19.1 per cent around the tetraploid range, the cells are derived from a relatively rapidly dividing cell population.

The presence of cell aggregates in the cytologic sample may at times cause difficulties in the evaluation of a specimen, especially in those cases exhibiting macronucleoli. A large nucleolus reflects the active engagement of the cells in protein synthesis thus occurring either in benign regenerative processes or in neoplasia. Close scrutiny however of the arrangement of the cells in sheets, of a delicate chromatin pattern and of the thread-like structures visible between the nuclei in some cases, will help in the differentiation with malignancy.

SUMMARY

The rate of occurrence of tissue repair in the cytologic sample from the female genital tract was determined by computerized evaluation of the cyto- and histo-pathology file of this institution, and results correlated with clinical findings.

The cytologic features of tissue repair were described on Papanicolaou stained material and the Masson's trichrome technique used for detection of elements deriving from the connective tissue. In Feulgen stained material, the DNA content was assessed by means of cytophotometry.

The possible etiology of cells deriving from tissue repair and the differential diagnosis with malignancy are discussed.

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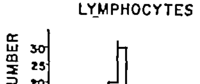
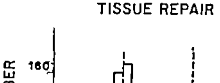


FIG. 7 Frequency distribution of DNA content in Feulgen-stained human lymphocytes measured for standardization of cells from tissue repair



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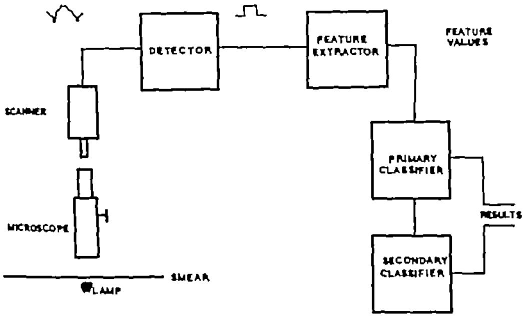

FIG 1 Diagram of proposed cell classification system For cervical smear prescreening the classifier result for each object increments one of series of counters. These counters accumulate the total umber of each cell type tha have been found in the smear

A block diagram of the simulated system is shown in Figure 1 The smear material is presented as a series of fields, each containing numerous cells (typically 10–100) Every field in turn is traversed as shown in Figure 2 by the scanner beam, which produces varying electrical output signal related to the optical density of the elemental field area currently under the beam The detector converts this continuously varying analogue waveform into a binary signal according to some algorithm which decides whether or not the current element is part of nucleus The output from the detector is therefore binary "1 signal whenever the scanner beam is traversing dense region The feature extractor collects detected elements which are geographically adjacent to one another so forming part of single object, and measures or calculates the features of each object which will be used in the classification procedures to follow

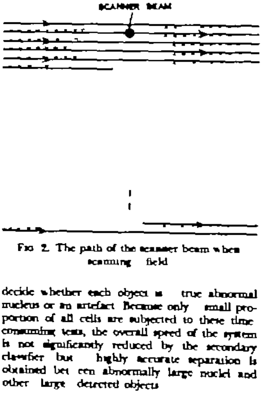

FIG 2 The path of the scanner beam when scanning field

Classification of detected objects is carried out in two stages in order to combine the speed advantage of single-feature method with the accuracy of multi-feature method The primary classifier performs quick, simple single-feature test to eliminate and roughly classify those detected objects which are obviously not abnormally large nuclei The few remaining detected objects are passed on to the secondary classifier where more complex and accurate tests are carried out to decide whether each object is true abnormal nucleus or an artefact Because only small proportion of all cells are subjected to these time consuming tests, the overall speed of the system is not significantly reduced by the secondary classifier but highly accurate separation is obtained between abnormally large nuclei and other large detected objects

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Automatic Detection and Classification of Cells in Cervical Smears

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A general description has been given in a previous paper[9] of a digital computer simulation system for investigating biological image analysis methods. This paper describes experiments with the system to evaluate various methods for detecting and classifying each cell in typical cervical smear fields.

Previous cervical smear pre-screening machines have usually been designed to detect individual abnormal cells in a smear which show characteristics indicative of carcinoma in situ, on the basis of the nuclear (and sometimes the cytoplasmic) characteristics of each cell. The Cytoanalyser[8] measures the maximum vertical chord and total light absorbtion of all objects in the smear with a density above a preset threshold value. Any objects which show unusual parameter values are considered as possibly abnormal cell nuclei, and smears containing such objects are indicated as requiring further investigations. A more recent development, the Vickers Cytology Screening Apparatus,[11] scans exfoliated cervical cells laid out on a long transparent flexible tape, the scanning machine again detects areas with density greater than a certain preset value for which the measured vertical chord is greater than a predetermined length. The density and length thresholds are adjusted so that the positions of cell nuclei with an unusually large area are indicated to the cytologist as requiring further investigation. The Rapid Cell Spectrophotometer[3] makes use of both nuclear and cytoplasmic characteristics to analyze cervical smear cells. The integrated optical density of each cell is measured at two wavelengths, in order to estimate its size and nucleic acid content. The results for all detected objects in a smear are displayed on a cathode ray tube enabling the cytologist to rapidly assess the normality of the smear.

A number of difficulties have arisen during clinical trials with these machines. Perhaps the major problem that has been encountered has been that of obtaining a good detection accuracy for abnormal cells, while at the same time reducing the number of "false positive" alarms to a satisfactory level. These false positive signals are usually caused by objects such as overlapped epithelial cell nuclei, groups of closely packed leukocytes, and overlapped cytoplasmic fragments, for which the parameters measured by the machine (such as area or integrated optical density) may be very similar to the parameters measured for true abnormal cell nuclei. The frequency of such artefacts is strongly dependent upon the technique used to deposit and stain the cervical smear material before analysis, and recently special preparation methods have been developed[2,3] which greatly reduce the number present in normal smears. In spite of these improvements, however, the necessary reduction in false positive signals has yet to be fully achieved.

Apart from the machines which have been designed specifically to aid the routine screening of large volumes of cytologic material, other more general systems have been developed which analyze and classify individual cells selected by the operator.[7] [9] These make use of the logical power available in a digital computer to carry out complex and highly accurate classification procedures which cannot be economically implemented in special-purpose electronic circuits. Although these systems are substantially slower in operation than the machines outlined above, the high resolution of cell feature measurements and multi-feature classification procedures used enable very accurate cell analyses to be achieved.

The cervical cell analysis system which has been simulated in the present work attempts to combine the advantages of the two types of system outlined above, in order to minimize the false positive abnormal cell signals obtained during the rapid analysis of cervical smear fields.

METHOD

Cell Analysis Procedure

Recent work on the visual analysis of cervical smears[4] has shown that smears from patients with carcinoma in situ of the uterine cervix contain a significantly greater number of abnormally large nuclei than do normal smears. The cell analysis system simulated in the present experiments is therefore designed to reliably detect abnormally large nuclei while rejecting other non-nuclear objects of similar size, so that an assessment of the normality of the smear can be made.

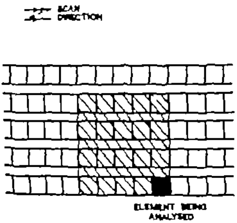

Fig 3 Neighborhood region for threshold calculation The threshold for each element is calculated from the average intensity of elements the neighborhood region (shaded) above and behind the element.

area is obtained with this algorithm The great majority of cell nuclei are correctly detected, even when enveloped in dense cytoplasm, and the boundaries of the cell nuclei agree well with the boundaries ascribed upon visual inspection Minor errors are, however caused by the "shadowing" of cell nuclei due to the rise in background density as the neighborhood *region traverses* the nucleus itself This effect may be eliminated by modifying the algorithm to replace the true element density *with* the current threshold value in all further averaging calculations whenever detected area is entered, so that the threshold value *is frozen* at its current level This modification leads to noticeable improvement in the detection of closely adjacent cell nuclei

Figure 4 shows (a) an original field photograph, (b) the corresponding computer display ( ) and (d) the displays when the areas detected by the fixed and modified variable threshold detection algorithms are deleted

Primary Classifier

The separation of normal nuclei from abnormally large detected objects by the primary classifier must be based upon small number of easily measured parameters if the required high analysis speed is to be obtained Parameters such as the measured area, integrated optical density maximum chord length or peak optical density of detected objects are potentially suitable for this purpose but the distribution data obtained with the Fidac scanner is unsuitable for tests involving absolute optical density values, because the density range is normalized for each individual photographed field according to the peak white and black areas observed Most of the current experiments have been carried out using the *measured area of each detected object* as the basis for primary classification, since this parameter provides higher resolution for a given element size and less susceptibility to scanner noise, than that obtained with linear measurements

An estimate of the maximum classification accuracy obtainable with this parameter was made as follows Using the modified variable threshold detection method with constants adjusted to give optimum detection of cell nuclei, the measured area of each detected object was listed Simultaneously *the reconstructed image of the field* (with numbered crosses superimposed to indicate object positions) was used in conjunction with the original field photographs to visually classify each object into one of the following categories

cytoplasmic fragment
leukocyte
superficial squamous cell nucleus
intermediate, basal or endocervical cell nucleus
overlapped nuclei

Histograms of the measured area for objects in each class were then plotted order to find the optimum class boundaries, and the values obtained stored in the primary classifier simulation routine Finally each field was reprocessed to obtain the optimum classification accuracy for the experimental data available

Secondary Classifier

The secondary classifier is designed to reduce the number of false "abnormal nucleus" signals caused by non-nuclear objects Three tests have been developed for this purpose

The first test, known as the projection test, consists of comparison between the vertical and horizontal projections of large objects (and b in Fig 5a) Objects which show an excessive difference between *the two measured projections* are discarded as non-nuclear objects, since the majority of abnormal nuclei exhibit relatively small degree of elongation

The area test eliminates highly irregular objects from the abnormal cell group In this test, the measured area is compared with the area of the box formed by the horizontal and vertical projections of the object (Fig 5b) If the measured area is substantially lower than the rectangular area, then the object cannot be round or elliptical, and so is more likely to be *non-nuclear object* than true abnormal nucleus

Finally the perimeter test detects large-area *objects which have a highly indented outline* The horizontal and vertical projections of the object outline (c and (d +) in Fig 5c) are compared

Cervical Smear Fields used in Simulation Experiments

The simulation experiments described below have been carried out with thirteen different fields from four separate cervical smears. Nine of the fields, taken from three normal smears, contain mainly superficial squamous cells, intermediate squamous cells and leukocytes. The remaining four fields form part of a smear containing many abnormal cells consistent with carcinoma in situ of the uterine cervix (this suspicion was later confirmed histologically) together with normal squamous cells and leukocytes.

The smears used for the experiments were prepared by the conventional method of directly smearing the spatula used to scrape the cervix onto a microscope slide, and staining the cells by the Papanicolou method. However the fields used have been selected to provide a cell distribution similar to that obtained with the modified specimen preparation methods mentioned above. In all, the data fields contain a total of approximately 480 cells.

Digitization of picture information for input to the computer system is presently carried out on the Fidac flying spot scanner[8] which scans a photographic negative of each field as shown in Figure 2, and measures the optical density of the elements as one of eight grey levels. With the magnification used in the current investigations, each element corresponds to a smear area of approximately 1 square micron. Reconstruction of the digitized fields on the computer display shows little evidence of shading between the different areas of any one field.

Cell Nucleus Detection

The accurate delineation of nuclear boundaries is a primary requirement in any cell classification system based upon the morphology of the cell nucleus. The computer simulation program enables the accuracy of detection algorithms to be visually assessed by displaying on a graphical display unit a reconstruction of each data field with a superimposed diagram of the detected areas. Two nuclear detection methods have been evaluated in this way.

The first method, the "fixed threshold" detection method has been used extensively in previous cervical smear analysis systems. The recorded density of each picture element is compared with a preset threshold value, and any elements with an optical density greater than this threshold are assumed to constitute part of a cell nucleus. The threshold density is set to the maximum value which allows all nuclei to be detected.

Several problems are evident from the computer displays produced with this algorithm. Most fields contain areas of overlapped cytoplasm which are considerably denser than the majority of cell nuclei, making it impossible to select a density threshold which at once accepts all nuclei, while at the same time rejecting all non-nuclear areas. Furthermore the detected area of many small, less dense cell nuclei is very much smaller than the area ascribed to the nucleus upon visual inspection, because of the blurring of nuclear boundaries caused by the finite rise time of the flying spot scanner and the loss of definition in the photographic processes. This blurring also gives rise to large variations in the measured area of cell nuclei as the threshold is adjusted.

The second nuclear detection method, the variable threshold method, operates by comparing the optical density of each picture element with a threshold that is automatically adjusted to compensate for variations of optical density of the background. The threshold is calculated as the sum of two component parts 1) a low fixed basic value and 2) a variable component proportional to the average density of the elements in a "neighborhood" region which is in close geographical proximity to the element under analysis. The constants of the algorithm are adjusted so that the gradual increase in density caused by cytoplasm appears as a variation in background, whereas the density change as a nucleus is entered is sufficiently rapid to permit detection. The picture signal is effectively differentiated by this process, but the spurious noise signals produced by simple differentiation are greatly reduced by the integration of densities in the neighborhood region. Hence the threshold used for the nucleus of cells such as leukocytes, which often have little or no visible cytoplasm surrounding the nucleus, is considerably lower than that used for a nucleus that is superimposed upon dense cytoplasm.

The neighborhood region shown in Figure 3 has been used in the simulation trials. This configuration is relatively simple to program in a digital computer and a good approximation may be implemented electronically with a small number of conventional components.

The density threshold Ta for element A is given by the expression

$$T_A = K_1 + K_2 \frac{\sum_{S_A} (D_x)}{N_A}$$

where

K_1 and K_2 are preset constants,

D_x is the optical density of any element x,

S_A is the neighborhood region set for element A

N_A is the number of elements in the neighborhood set

A much improved definition of nuclear bound-

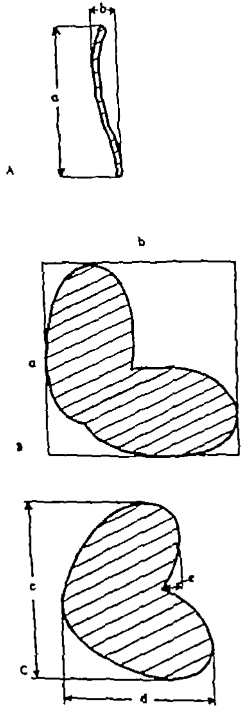

FIG. 5. Secondary classification tests for rejecting non-nuclear objects. (a) Projection test, which rejects horizontal and vertical elongated objects; (b) Area test which rejects non-elliptical objects; (c) Perimeter test, which rejects objects with indented outlines.

with the horizontal and vertical chord lengths of the object. Objects which exhibit a large discrepancy between these respective values are rejected.

Each of these tests has been applied to the objects noted as abnormal nuclei by the primary classifier. Values for the discrimination levels in each test were selected on the results of preliminary trials as

1) projection test: object rejected as non-nuclear if the difference between the maximum and minimum projection is greater than one-third of the maximum projection length.
2) area test: object rejected as non-nuclear if the measured area is less than one-half of the area of the projection box.
3) perimeter test: object rejected as non-nuclear if the difference between projection and perimeter projection is greater than one element in either direction.

RESULTS

Table I shows the area ranges determined for each class by the optimization process, and Table II details the classifications obtained using these ranges. Approximately 55 per cent of all nuclei visually classified as abnormal are classified by the system as "abnormally large" and 19 false positive entries in this group are obtained with 607 detected objects (~3%). Most of these false positive signals are caused by overlapping nuclei or by the inclusion of dense cytoplasm in the detected area of normal nuclei. Two reasons are apparent for the false negative abnormal cell classifications made by the system. Firstly, some cells are considered as abnormal upon visual examination even though the nucleus is not abnormally enlarged because of abnormalities such as irregular nuclear outline, uneven chromatin distribution in the nucleus, etc. Secondly, the measured area of many of the abnormally large nuclei is artificially depressed by the variation in absolute grey level values between the different

TABLE I. *Optimum ranges for Primary Classifier Area (Picture Elements)*

	Min	Max
Objects too small to be nuclei (S)	0	1
Leukocytes (Lk)	2	9
Superficial squamous cell nuclei (Sp. sq)	10	23
Deep layer squamous and endocervical cell nuclei (D. t. sq)	24	38
Malignant nuclei (Mal)	39	127
Objects too large to be nuclei (L)	128	∞

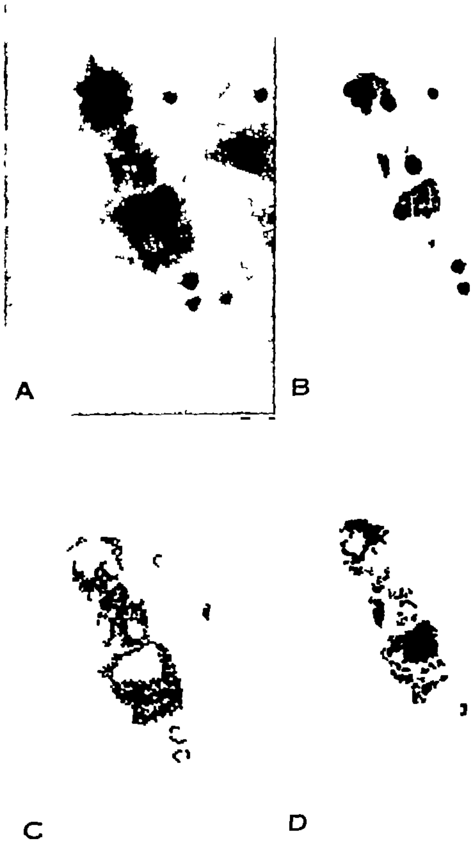

FIG. 4 Comparison of nuclear detection algorithms a) Section of original field photograph b) Corresponding basic computer display c) Fixed threshold detection, note the large areas of cytoplasm that have been detected d) Modified variable threshold detection most nuclei has been accurately outlined

secondary tests Whereas a group of abnormal cells is easily detected during visual analysis of a smear the accuracy of the automated classification system is generally highest for the individual abnormal cells which usually surround such group

The results obtained during this investigation show that the nuclear detection and classification procedures which have been simulated give significant improvement over other methods previously used. The detection rate for abnormal cells is, as yet, too low for useful prescreening system, but considerable improvement is expected when the difficulties in data presentation inherent in the present simulation system are removed The frequency of false abnormal cell signals, which has been the major difficulty encountered in previous cervical smear systems, has been significantly reduced by the use of the variable threshold detection method (which eliminates dense cytoplasmic regions) and the secondary classification procedures (which eliminate the majority of overlapped or touching nuclei, etc.)

It is important to consider any cervical smear analysis procedures designed for use in prescreening system in the context of the overall system Thus problems caused by dense cytoplasm may be diminished by the use of special staining and optical filtering techniques to improve the nuclear/cytoplasmic contrast, and cell disaggregation and dispersion procedures may be used to reduce the incidence of overlapped nuclei. It may be expected that the use of such procedures in combination with improved analysis methods such as those described above will enable some useful mechanical aid to the cervical smear screening process to be developed in the foreseeable future

SUMMARY

A computer image analysis system has been used to simulate cell classification methods for use in the automatic prescreening of cervical smears The procedures are designed to locate cells containing abnormally large nuclei, while t the same time reducing as far as possible the number of false "large nucleus alarms

The paper describes experiments in which photographs of cervical smear fields containing numerous exfoliated cells are used to evaluate procedures for the detection, measurement and classification of cell nuclei A detection procedure has been developed which gives a more accurate delineation of nuclear boundaries than that obtained with other methods previously used Cell nucleus classification is carried out in two stages firstly fast algorithm based on the area of detected regions is used to separate suspicious objects from those which are obviously not abnormally large nuclei, and secondly objects classified as suspicious are subjected to more accurate "secondary" tests which are designed to separate abnormally large nuclei from other large detected objects such as overlapped nuclei, leukocyte clumps and folded cytoplasm. Results are given which show that these secondary classification tests give considerable reduction in the number of false abnormal cell alarms

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8 Mendelsohn, M. L, et al Digital transformation and computer analysis of microscopic images.

TABLE II *Comparison of Visual and System Primary Classification*

	Class ascribed visually					
	Cytoplasm	Leuk	Sp sq	Int sq	Mal	Overlapped nuclei
Cy	56	29	9	2	0	0
Lk	25	108	62	6	2	2
Sp sq	7	39	109	26	5	1
Int Sq	0	2	17	19	16	6
Mal	2	0	3	3	30	11
L	3	0	0	0	0	7
	93	178	200	56	53	27
			Total 607			

TABLE III *Efficiency of Secondary Classification Tests*

	Number Detected	Failed Perim	Failed Proj	Failed Area	Passed All
Cytoplasm	2	1	1	1	1
Leukocytes	0	—	—	—	—
Sp. sq nuclei	3	2	0	1	1
Int. sq nuclei	3		0	0	1
Mal. nuclei	30	8	8	0	18
Overlapped nuc	11	9	5	3	

fields, because the maximum optical density of nuclei in the malignant smear fields is considerably higher than that in the normal fields.

The number of objects correctly classified by the system, although adversely affected by the characteristics of the picture data used in the experiments, demonstrates that the area classification algorithm is sufficiently accurate to yield useful cytologic information on normal smears such as the number of squamous cells and incidence of leukocytes.

Table III shows the number of large detected objects which are rejected as non-nuclear by each secondary test. The area test results are somewhat inferior to those from the other two tests, but this may be due to a slight error in the discrimination level setting. (The number of large objects available is insufficient to permit the reliable use of a mathematical optimization technique for these levels.)

The variable threshold detection algorithm gives more outline irregularities in the detected area for abnormal nuclei than does the simple threshold algorithm, because of the changes in threshold across the nucleus which sometimes occur due to density variations in the cytoplasm surrounding the nucleus. These irregularities sometimes cause erroneous rejection of a true abnormal cell nucleus by the perimeter test. Other false negative abnormal cell classifications are caused by the detection of two abnormal cell nuclei in close proximity as a single object (leading to rejection of the object by all three secondary tests) and to the expected rejection of nuclei exhibiting a highly deformed morphology.

DISCUSSION

Overall System Performance

The overall performance of the complete cell analysis system with the test data fields is summarized in Table IV. These results are obtained using the modified variable threshold area detection method, the area primary classification algorithm, and all three secondary classification tests.

The secondary tests provide a very significant reduction in the number of false positive abnormal cell signals from the normal smear fields. Without the secondary tests, false positive signals are caused predominantly by overlapped nuclei (six signals) and cytoplasmic fragments (four signals). The secondary tests eliminate all but one of these false positive signals, the one remaining being caused by two overlapped intermediate squamous cell nuclei which are orientated in such a way that the object passes all three secondary tests. The other false positive signals are obtained from squamous cell nuclei surrounded by dense cytoplasm for which the detected area is just inside the range for abnormal cells.

The false negative signals arise from two main causes: 1) the erroneous classification of malignant cell nuclei as normal squamous cells, due to errors in the primary classification procedures, and 2) rejection by the secondary tests as described above. Some erroneous classifications of the first type are inevitable in any system based on single-parameter classification, since it is most unlikely that all cells in a smear which are classified as abnormal by multi-parameter visual analysis will violate any one single criterion for abnormality. The frequency of errors of the second type is dependent on the number of cell overlaps that occur in the data fields (i.e. upon the smear preparation method) and by the efficiency of

TABLE IV *Overall Results*

	Without secondary tests	With secondary tests
Normal smears		
No. of nuclei present	320	320
No. of objects detected	340	330
No. of nuclei correctly classified	303	303
No. of false positive signals	12	
Malignant smear		
No. of abnormal nuclei present	53	53
No. of abnormal nuclei correctly classed	30	18

secondary tests. Whereas a group of abnormal cells is easily detected during visual analysis of a smear the accuracy of the automated classification system is generally highest for the individual abnormal cells which usually surround such group.

The results obtained during this investigation show that the nuclear detection and classification procedures which have been simulated give a significant improvement over other methods previously used. The detection rate for abnormal cells is, as yet, too low for a useful prescreening system, but considerable improvement is expected when the difficulties in data presentation inherent in the present simulation system are removed. The frequency of false abnormal cell signals, which has been the major difficulty encountered in previous cervical smear systems, has been significantly reduced by the use of the variable threshold detection method (which eliminates dense cytoplasmic regions) and the secondary classification procedures (which eliminate the majority of overlapped or touching nuclei, etc.)

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Computer Recognition of Cells from Asymptomatic Lymphocytic Leukemia I Methodologic Study

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Recent developments in objective image evaluation, and their application to tumor cell identification, have shown that machine recognition of cells can be accomplished with a high degree of consistency and reliability The objective methods result in a final numerical value such as likelihood ratio, for the correctness of the diagnostic decision as it applies to an individual cell.

Cellular material from the hematopoietic system has previously been used to test number of algorithms for the assessment of image texture,[10] and to test portions of non-supervised learning program for cell recognition It is the purpose of this paper to describe the application of self-learning computer program[1] to the recognition of cells from case of asymptomatic chronic lymphocytic leukemia

A case with cells clearly exhibiting the typical appearance of cells found in this disease was selected This was done in keeping with the intent of this methodologic study to establish which cell image properties discriminate these cells from normal lymphocytes, the likelihoods for correct recognition that can be expected in an ideal case from different textural measures, and the variances found within material from single patient It should furthermore render information about the consistency of the classification algorithm which is established by the program on a training set of cells by applying it to an object set, f cells from the same patient, but not included in the training set The material should also give an indication whether non-supervised learning programs can be gainfully employed in the assessment of the homogeneity of cell populations as used here

It is understood that clinical diagnostic decisions are always based on the assessment of substantial sample from the cell population on a slide It rests its conclusions greatly upon the number of cells with abnormal appearance Conclusions of this type are sought from the non-supervised learning section of the program The supervised learning section is aimed at the certainty of identification of individual cells only

Materials and Methods

Whole blood was collected in EDTA, immediately spun down, and the buffy coat removed Thin smears were made, fixed in alcohol for 15 minutes, washed in tap water and air dried The preparations were then stained with hematoxylin after Mayer Bennet

A total of 200 lymphocytes, 100 each from normal human donor and from a case of asymptomatic chronic lymphocytic leukemia were measured Measurements were carried out on the Cytoscan microscope with measuring spot size of 0.5 × 0.5 μ^2 at 570 nm The cell images were divided into pair of training sets of 50 cells each to be used in the supervised learning section of the TICAS Master Program to establish classification algorithm, and 100 cells (50 from each case) to be used as an object set and to be classified by the subprogram CLASIFY according to the above-mentioned classification algorithm One hundred randomly selected cells, 50 from each case were then entered as data into the non-supervised learning section of the master program

All cell images were cleaned by the TICAS 8 program prior to further processing

Case Histories

The selected case of chronic lymphocytic leukemia is 59 year old male who is totally asymptomatic The complete blood count data are hemoglobin 13.1 gm hematocrit 40 vol%,

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Carl Zeiss, Oberkochen Germany

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CASE HISTORIES

The selected case of chronic lymphocytic leukemia is 59 year old male who is totally asymptomatic. The complete blood count data are hemoglobin 13 1 gm%, hematocrit 40 vol%,

---

Carl Zeiss, Oberkochen, Germany

white blood cells, 26 000 The differential count yielded bands 2 segmented leukocytes 24 lymphocytes 72 eosinophils 1 monocytes 1 The bone marrow morphology report indicated an early chronic lymphocytic leukemia. No palpable lymph node enlargements were present. The normal case was a 26 year old male with no known illness. The white blood cell count was 11 100

RESULTS

The cells from asymptomatic chronic lymphocytic leukemia (ACLL) appeared visually to be smaller and to have a coarser chromatin pattern in the nucleus than the cells from a normal lymphocytic population (LNRM) Figures 1 and 2 show examples.

This is clearly reflected in the histogram of partial extinction values found in the two cell samples (Fig. 3) and in the values observed for a number of cell image properties as shown in Table I with their respective variances and coefficients of variation (C.V.) The supervised learning section evaluated a total of 57 different image properties These included 18 values for relative frequency of occurrence of gray values (histogram data) six values assessing global data as given in Table I 22 values expressing transition probabilities between different gray value ranges, and 11 values expressing the cells extinction profile. For this latter measure the cell images were converted to polar coordinates and gray values averaged as a function of cell radius

The cells from the training sets were classified as shown in the classification matrix applying to LINCOMB 5, the composite final discriminator formed by the program

	LNRM	ACLL
LNRM	50	—
ACLL	1	49

with an average likelihood for the value of LINCOMB 5

	ACLL	LNRM
LR LINCOMB 5	$9\ 03 \times 10^{4}$ 1	$5\ 82 \times 10^{4}$ 1

The high values found for the average classification likelihood expresses the great certainty with which the program, in this typical case, recognizes a cell as being not normal. It approaches the very high likelihoods with which human observers identify well known visual patterns.

The program was operated in a subprogram sequence which first selects a number of uncorrelated image properties, and forms a linear combination of them for each reducing subroutine. The resulting linear combinations from the different subroutines are then subjected to the same procedure, and a final, composite descriptor (LINCOMB 5) is formed, on the basis of which the above given likelihood ratios were computed.

The linear combinations LINCOMB 5 of the selected descriptors tend to be normally distributed The training set of cells from the ACLL and LNRM cases, respectively showed distribution means and standard deviations as follows

	$\bar{x}$	s
ACLL	−0 1233	0 03741
LNRM	+0 0096	0 01414

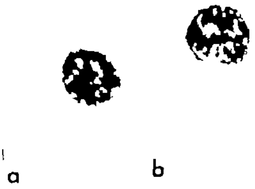

Fig 1

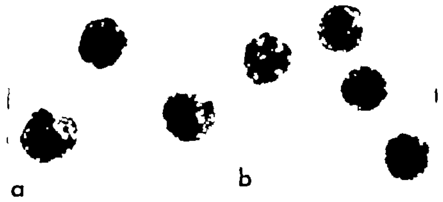

Fig. 2

The corresponding distributions are shown in Figure 4

The program placed the decision boundary at the point LINCOMB 5 = 0 030. This leads to the following expected classification errors

	LNRM	ACLL
LNRM	99 74	0 26
ACLL	0 64	99 36

It must be understood that both distributions represent but one case each, and therefore, no conclusion can be drawn as to the distribution of LINCOMB 5 in a population of cells drawn from many different patients Although a separation of the two distributions is still to be expected, the error rates will become higher and the average likelihood for classification as ACLL, or LNRM will decrease

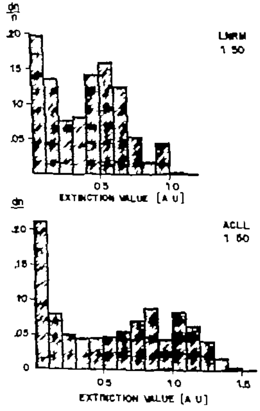

Fig 3

The following descriptors entered into the linear combination LINCOMB 5 The program selected from the histogram data the relative frequency of occurrence of values in the interval from 1 00 to 1 09 relative extinction units (HIST 11) and from 0 70 to 0 79 (HIST 8) with the former carrying greater relative weight The histogram data alone resulted in the classification matrix

	LNRM	ACLL
LNRM	49	1
ACLL	0	50

with an average likelihood ratio of

	ACLL	LNRM
LR LINCOMB 1	4 6 × 10[illegible] 1	7 6 × 10[illegible] 1

The subroutine assessing transition probabilities yielded two transitions with good discriminatory power These are transitions from the extinction value range of 0 25 to 0 49 into the same range (ROW 2, 2) and between the ranges 0 75 to 0 99 and 1 00 to 1 24 (ROW 4 5) The average likelihoods resulting from linear combination LINCOMB 3 of these two image properties were

	ACLL	LNRM
LR LINCOMB 3	1 34 × 10[illegible] 1	1 45 × 10[illegible] 1

The extinction value profile as function of cell radius (PAVG) essentially measures cell size, roundness and average extinction It was found that significant differences between the examined cell samples existed for the values at distance of 3 and 7 measuring spot diameters *removed from the* cell center both carrying about equal weights.

TABLE I

	ACLL			LNRM		
	X̄		CV %	X̄		CV %
Relative cell area	159 5	25 33	15 8	294 3	44 5	15 2
Integrated extinction	97 19	5 30	5 4	114 28	6 04	5 3
Averaged extinction	0 612	0 064	10 5	0 391	0 054	13 9
Relative area of cytoplasm in	34 6	7 42	20 4	40 1	8 11	21 0
Relative area of nucleus [illegible]	65 4	7 94	11 8	59 9	9 49	15 0
N/C ratio	1 [illegible]	0 62	31 4	1 49	0 69	43 4

white blood cells, 26 000 The differential count yielded bands 2 segmented leukocytes 24 lymphocytes 72 eosinophils 1 monocytes 1 The bone marrow morphology report indicated an early chronic lymphocytic leukemia. No palpable lymph node enlargements were present. The normal case was a 26 year old male with no known illness. The white blood cell count was 11 100

RESULTS

The cells from asymptomatic chronic lymphocytic leukemia (ACLL) appeared visually to be smaller and to have a coarser chromatin pattern in the nucleus than the cells from a normal lymphocytic population (LNRM) Figures 1 and 2 show examples

This is clearly reflected in the histogram of partial extinction values found in the two cell samples (Fig 3) and in the values observed for a number of cell image properties, as shown in Table I with their respective variances and coefficients of variation (C.V.) The supervised learning section evaluated a total of 57 different image properties These included 18 values for relative frequency of occurrence of gray values (histogram data) six values assessing global data as given in Table I 22 values expressing transition probabilities between different gray value ranges, and 11 values expressing the cells extinction profile. For this latter measure the cell images were converted to polar coordinates and gray values averaged as a function of cell radius

The cells from the training sets were classified as shown in the classification matrix applying to LINCOMB 5, the composite final discriminant formed by the program

	LNRM	ACLL
LNRM	50	—
ACLL	1	49

with an average likelihood for the value of LINCOMB 5

	ACLL	LNRM
LR LINCOMB 5	$9\ 03 \times 10^{4}$ 1	$5\ 82 \times 10^{4}$ 1

The high values found for the average classification likelihood expresses the great certainty with which the program, in this typical case, recognizes a cell as being not normal. It approaches the very high likelihoods with which human observers identify well known visual patterns.

The program was operated in a subprogram sequence which first selects a number of uncorrelated image properties, and forms a linear combination of them for each reducing subroutine. The resulting linear combinations from the different subroutines are then subjected to the same procedure, and a final, composite descriptor (LINCOMB 5) is formed, on the basis of which the above given likelihood ratios were computed.

The linear combinations LINCOMB 5 of the selected descriptors tend to be normally distributed The training set of cells from the ACLL and LNRM cases, respectively showed distribution means and standard deviations as follows

	$\bar{x}$	s
ACLL	−0 1433	0 03741
LNRM	+0 0096	0 01414

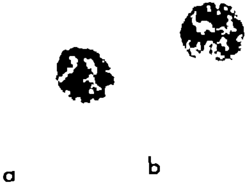

Fig 1

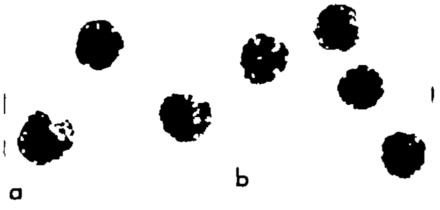

Fig. 2

The corresponding distributions are shown in Figure 4.

The program placed the decision boundary at the point LINCOMB 5 = 0.000. This leads to the following expected classification errors:

	LNRM	ACLL
LNRM	97.74	0.26
ACLL	0.64	99.36

It must be understood that both distributions represent but one case each, and therefore, no conclusion can be drawn as to the distribution of LINCOMB 5 in a population of cells drawn from many different patients. Although separation of the two distributions is still to be expected, the error rates will become higher and the average likelihood for classification as ACLL or LNRM will decrease.

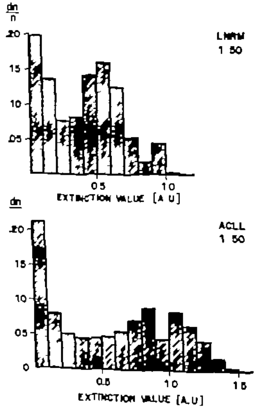

Fig 3

The following descriptors entered into the linear combination LINCOMB 5. The program selected from the histogram data the relative frequency of occurrence of values in the interval from 1.00 to 1.09 relative extinction units (HIST 11) and from 0.70 to 0.79 (HIST 8) with the former carrying greater relative weight. The histogram data alone resulted in the classification matrix

	LNRM	ACLL
LNRM	49	1
ACLL	0	50

with an average likelihood ratio of

	ACLL	LNRM
LR LINCOMB 1	4.6 × 10[illegible] 1	7.6 × 10[illegible] 1

The subroutine assessing transition probabilities yielded two transitions with good discriminatory power. These are transitions from the extinction value range of 0.25 to 0.49 into the same range (ROW 2, 2) and between the ranges 0.75 to 0.99 and 1.00 1.24 (ROW 4, 5). The average likelihoods resulting from the linear combination LINCOMB 3 of these two image properties were

	ACLL	LNRM
LR LINCOMB 3	1.31 × 10[illegible] 1	1.45 × 10 1

The extinction value profile as a function of cell radius (PAVO) essentially measures cell size, roundness and average extinction. It was found that significant differences between the examined cell samples existed for the values at a distance of 3 and 7 measuring spot diameters removed from the cell center, both carrying about equal weights

TABLE I

	ACLL			LNRM		
	$\bar{x}$		CV %	$\bar{x}$		CV %
Relative cell area	159.9	25.33	15.8	204.3	44.5	15.2
Integrated extinction	97.19	5.30	5.4	114.28	6.04	5.3
Averaged extinction	0.612	0.064	10.5	0.391	0.054	13.9
Relative area of cytoplasm (in %)	34.6	7.42	20.4	40.1	9.11	21.0
Relative area of nucleus (%)	65.4	7.94	11.8	59.9	9.40	15.0
N/C ratio	1.89	0.62	31.4	1.49	0.69	43.4

white blood cells, 26 000 The differential count yielded bands 2 segmented leukocytes 24 lymphocytes 72 eosinophils 1 monocytes 1 The bone marrow morphology report indicated an early chronic lymphocytic leukemia. No palpable lymph node enlargements were present. The normal case was a 26 year old male with no known illness. The white blood cell count was 11 100

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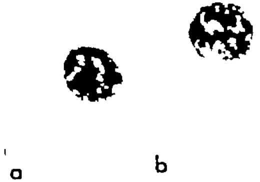

FIG 1

The cells from the training sets were classified as shown in the classification matrix applying to LINCOMB 5, the composite final discriminant formed by the program

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LNRM	50	—
ACLL	1	49

with an average likelihood for the value of LINCOMB 5

	ACLL	LNRM
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The high values found for the average classification likelihood expresses the great certainty with which the program, in this typical case, recognizes a cell as being not normal. It approaches the very high likelihoods with which human observers identify well known visual patterns.

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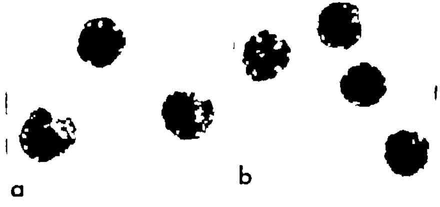

FIG 2

The good agreement with the data obtained from the object set applies as well to means and variances found for the individual, evaluated image properties. Excellent consistency of the procedure was found.

Non-Supervised Learning Section

One hundred cell images were submitted to a non-supervised learning program to test the feasibility of detection of subsets consisting of 50 normal and 50 leukemic cells each. No automatic feature selection program was applied. It was obvious that already some of the global descriptors, as shown in Table I would clearly separate the normal from the leukemic cells. For this reason, only textural measures which reflected the statistical property of the gray value ensemble were selected.

By normalizing the value distributions in each cell image to unity the potential of the non-supervised learning program could thus be evaluated independent of image measures depending strongly on total stain uptake, or cell size. Employed were data from the histogram subroutine and the transition probability measure between two consecutive line scan values, normalized for the entire transition probability matrix.

The non-supervised learning program applied here has been briefly described earlier. It first forms groups of neighbors and computes local densities for each such group. These densities enter into an association value computed for pairs of cells. Cells are then grouped in an agglomerative, binary sequence in order of decreasing association. The resulting association order represents a tree graph. The program has provisions to enforce formation of an open graph.

The association graph is shown in Figure 5. It serves to trace similarities between small subgroups of cells and to permit comparison with their recorded photomicrographs.

A different representation of the existing associations between cells in the entire set is given in Figure 6. Each vertical line in this graph represents a cell. The height of each line represents the degree of association with other cells. A cell which, for example would have only a line of minimum height would appear very late in the grouping sequence having a low association. A cell represented by a line of great height is grouped early in the sequence, being highly associated with its neighbors.

This graph shows rather clearly the existence of two large subsets of cells, of which the left one appears slightly less homogeneous. It contains the leukemic cells. The right mode is formed by the normal cells. Seven normal cells appear in the first mode and two leukemic cells in the second mode, which might serve to estimate the diagnostic

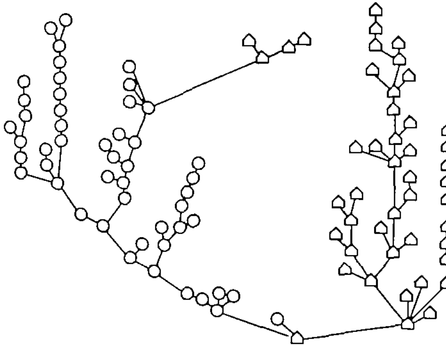

Fig. 6

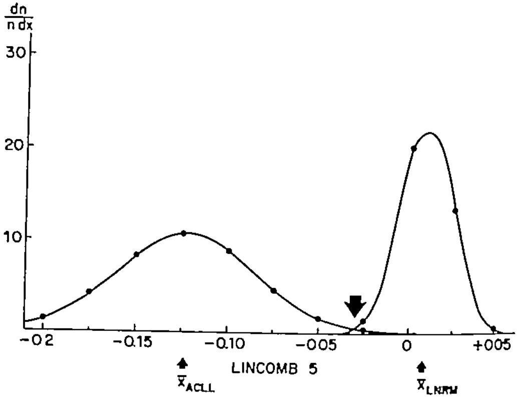

Fig 4

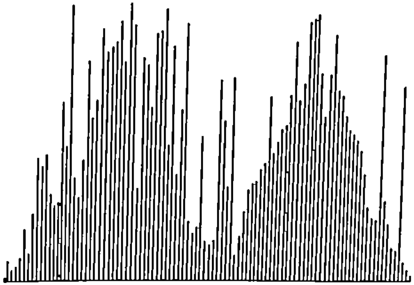

Fig 5

The average classification likelihood turned out to be high again, as

	ACLL	LNRM
LR LINCOMB 4	4.56×10^{14} 1	3.48×10^{4} 1

Object Set Data (testing of the classification algorithm)

The decision sequence and thresholds as derived from the training set data were next applied to 100 cells, 50 from each sample which had not been included in the training sets, to test the consistency of the classification algorithm. The composite discriminator LINCOMB 5 resulted in a classification matrix

	LNRM	ACLL
LNRM	49	1
ACLL	—	50

and the average classification likelihoods

	ACLL	LNRM
LR LINCOMB 5	2.04×10 1	2.03×10^{4} 1

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accuracy of the non-supervised learning routine. It should be pointed out again, though, that only *the selected small subset* of normalized image properties had been used for discrimination here.

DISCUSSION

The supervised learning section of the Master Program resulted in very high likelihoods for the recognition of cells as either normal, or leukemic. This confirms that the textural features of these typical cell images are well defined, and the conclusion is reflected by the coefficients of variation, *as listed for* the global data in *Table I*. It should be noted that these coefficients of variation are not noticeably greater in the set of leukemic cells, which is unusual for cells from malignant material. The extinction value histogram for the leukemic cells displays the *typical shift to higher values* frequently found in such material. As a whole, these leukemic cells constituted very good patterns for machine recognition. Testing for consistency of the algorithm on an object set resulted in a low error rate. This indicates that the observations on 50 cells each did not lead to a classification algorithm too closely tailored to the specifics of the *training set. It is not expected that a* comparably low rate of misclassifications can be maintained when data from the different patients are pooled and when data from different staining batches are compared. There is cause for optimism though that with some preprocessing acceptably low error rates can be maintained even under those circumstances.

Generally valid classification likelihoods for normal cells and blood samples from patients with asymptomatic lymphocytic leukemia have to be estimated from a substantial number of samples from many different cases. Such data are presently being collected and a data bank[6, 7] for leukemic cells has been initiated to have all information readily available for statistical evaluation and updating.

The non-supervised learning technique is clearly capable of detecting the occurrence of a subset of cells with different properties, with an acceptably low error rate. The particular subprogram employed here is one of the more powerful methods for the detection of such subsets, but it is computationally expensive.

The results *confirm earlier studies* which showed the usefulness of a transition probability measure for the recognition of leukemic cells[2]. The discriminating cell image properties could relatively easily be extracted by a small hybrid device attached directly to the *scanning* microscope and, for an established classification algorithm, almost immediate classification decisions could be obtained at moderate cost.

SUMMARY

The application of a self-learning computer program of TICAS is demonstrated on cells from asymptomatic chronic lymphocytic leukemia to show *which* cell image properties discriminate these cells from normal lymphocytes, and to show the variances found within material from individual patients.

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served with the same staining procedure and measuring equipment (but with extremely favorable material) an exact twofold increase of the measuring value for cells with tetraploid DNA content, as compared with diploids with a variation coefficient between nuclei of 1.6 and 2 per cent, respectively. Due to paucity of nuclei with an exact tetraploid Feulgen-DNA content in the nuclear population in pleura or peritoneum, a second Gaussian curve for this ploidy class could not be isolated. Therefore a tetraploid class with a mean of exactly two times the mean of the diploids was constructed, in which the same spread in arbitrary units was supposed to exist as had been found in the diploid nuclei. On an empirical basis, two times the calculated standard deviation of the values in the 2k group was taken as a measure for this spread; on a similar basis an 8k class was created. These classifications are highly arbitrary and do not fit any statistical technique, nor do they pretend to contain all but only a part of the nuclei in the different Feulgen-DNA classes concerned. Moreover, rather narrow limits (especially under limits) of the presumptive 4k and 8k group are important in view of our diagnostic system. It proved only a workable method for subdividing the population into classes, which do not overlap. If the mean of the supposed diploid group was found to be m and the standard deviation p, the classes were then calculated as follows:

a) class 2k (mainly diploid nuclei isolated as separate group from the curve): from m − 2p to m + 2p

b) class 4k (consisting of a large part of the nuclei with tetraploid Feulgen-DNA value): from 2m − 2p to 2m + 2p

c) class 8k (consisting of euploid or aneuploid nuclei of higher ploidy, not necessarily true octoploids): from 4m − 2p to 4m + 2p

d) classes 3k and 6k: intermediate values between 2k and 4k, and 4k and 8k, respectively.

Measurements were made in each smear on about 50 lymphocytes and 25 to 100 non-inflammatory cells (mesothelial cells and possible tumor cells). In each of these two groups the cells were measured at random, as they came into view under the microscope. Only undoubtedly single and undamaged nuclei were evaluated.

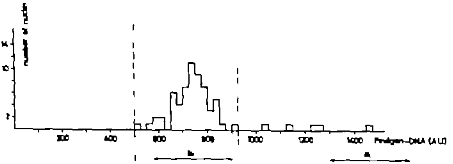

FIG. 1. Patient No. 11 (ascites due to backward failure). Frequency distribution histogram of Feulgen-DNA readings per nucleus (in arbitrary units = A. U.). *Mesothelial cells* in the fluid sample: mean of separated diploid values 735 ± 73 A. U. (between dotted vertical lines); limits of class 2k: 575–900 A. U.; limits of class 4k: 1300–1625 A. U. *Diagnosis*: only 1% of the mesothelial cells in class 4k or higher being found.

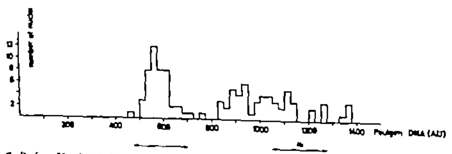

FIG. 2. Patient No. 32, fluid No. 15 (lymphatic leukemia with pleura involved). *Lymphocytes*: mean of separated diploid values 581 ± 50 A. U. Limits of class 2k: 475–700 A. U.; limits of class 4k: 1050–1273 A. U. *Diagnosis*: 21% of lymphocytes in class 4k or higher: lymphocytic malignancy.

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Tumor Diagnosis in Pleural and Ascitic Effusions Based on DNA Cytophotometry

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It is well established that the nuclei of malignant tumor cells generally show an increased Feulgen-DNA content, as compared with nuclei of non-malignant cells in the same tissue[2-11] exceptions seem to be very rare[6, 10] This finding corresponds with an increase in chromosomal numbers, which has been observed in malignant tumors[3, 11] For future diagnostic use Feulgen cytophotometry seems to hold more promise than chromosome counting and typing as a larger number of nuclei can be analyzed Modern developments in scanning photometry enable a quick determination of the Feulgen-DNA content of a nucleus We have been unable to find any information concerning the DNA content of cells obtained from benign or malignant pleural or peritoneal fluid which can be measured quite easily as it concerns a suspension of free cells. Only Atkin *et al* described the modal DNA value of cells in a single specimen of malignant ascitic fluid but they did not specify values for individual cells.

Some workers, aware of the possible diagnostic value of increased DNA contents, examined the Feulgen DNA content[20] or total nucleic acid content[3] in the nuclei of cervical smears These workers have compared their findings with the cytologic or histologic diagnosis in each case and concluded that the DNA content may be a useful diagnostic criterion for malignancy

In this paper the results of a cytophotometric investigation is reported, concerning the possibility of utilizing Feulgen-DNA values for diagnostic purpose in specimens of human pleural and ascitic fluids It should be pointed out that this investigation was not meant as more than a tentative investigation into the possible diagnostic value of Feulgen cytophotometry with this material especially with regard to the group of suspect and false-negative cases, which is remarkably large [21]

Material and Methods

A total of 77 specimens of pleural and 10 of ascitic fluids was examined, obtained chiefly from the Department of Chest Diseases in the Wilhelmina Gasthuis, University of Amsterdam

Routine cytologic examination was performed independently of the cytophotometric research project The Papanicolaou-stained slides were examined by the chief-cytotechnician and the head of the cytopathologic laboratory

A small heparinized portion of the specimen (25–50 ml) was separated for cytophotometric investigation and examined within 3 hours of collection of the fluid After centrifugation at 800 r p m smears were made from the sediment, which were fixed in 4% formaldehyde with 7½% sucrose, stained with a Feulgen-technique for quantitative purposes as described elsewhere[7] and mounted in Euktt. Measurements were made on a slightly modified scanning and integrating cytophotometer according to Deeley (Barr & Stroud, Glasgow) with a digital voltmeter at 560 nm using a 45 × oil immersion objective

Each nucleus was measured three times the sum of these readings was taken as the result Under these conditions, epithelial cells in smears from oral mucosa and mesothelial cells from freshly obtained non-malignant surgical specimens were found to have a Feulgen-DNA content of about 700 to 800 Arbitrary Units (A U) while lymphocytes in the peripheral blood had a value of about 500 A U

In each sample the values measured were plotted as a frequency-distribution histogram with units of 25 A U of Feulgen-DNA The mean from the diploid nuclei was calculated by isolating that part of the histogram which approached a Gaussian distribution and had a Feulgen-DNA value in the expected range of 600–800 A U all peripheral values were excluded (Fig 1) The reason for this somewhat extraordinary procedure for obtaining a diploid standard is that the standard cannot be taken but from the sample itself This is due to various degrees of cellular lysis in pleural and ascitic fluids, which will be accompanied by varying degrees of breakdown I the nuclear DNA content,[1] combined with variation due to different degrees of aneuploidy One of us has ob-

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Table I Control Samples

	No	Per(itoneum) Pl(eura) B(uccal mucosa)	S(urgery) A(utopsy)	number of measured nuclei	mean diploid Feulgen DNA value ± S D (in arbitrary units)	percentage of nuclei in DNA class						
						<2k	2k	3k	4k	6k	≥8k	≥16k
TUMOR-NEGATIVE	F 210	Per	S	100	[illegible] ± 34	—	[illegible]	—	2	—	—	2
	F 211	Per	S	100	[illegible] 41	1	98	1	—	—	—	—
	F 212	Pl	A	100	[illegible] 43	2	[illegible]		5	—	—	5
	F 13	Per	A	100	[illegible]	1	[illegible]	—	—	—	—	—
	F 21	Pl	A	00	[illegible] 42	—	95	5	—	—	—	—
	F 215	Pl	S	80	[illegible] 53	—	100	—	—	—	—	—
	F 216	Pl	S	50	[illegible]	—	100	—	—	—	—	—
	21	Pl	S	100	794 [illegible]	—	[illegible]	3	1	—	—	1
	F 218	Per	A	50	[illegible] 35	—	100	—	—	—	—	—
	F 219	Pl	S	00	719 [illegible]	—	97	2	1	—	—	1
	220		—	100	16 5	1	99	—	—	—	—	—
	F 221	B	—	80	[illegible]	—	[illegible]	2	—	—	—	—
	F 222	B	—	50	[illegible]	3	97	—	—	—	—	—
TUMOR POSITIVE	F 83	ascites due to metastasized carcinoma of the ovary	A	63	905 44	—	82		8	5	2	15
	F	pleural fluid due to metastasized lung cancer		100	[illegible] 53	—	40			30	17	7

Discussion

No author appears to have yet reported DNA measurements or chromosome counts in normal mesothelial cells. The results summarized in Table I provide evidence that mesothelial nuclei are basically diploid. This means that mesothelial nuclei can have not more than twice the diploid DNA content in the premitotic G2-phase. A Feulgen-DNA value higher than tetraploid therefore should indicate nuclei of extra-mesothelial origin, which in case of thoracic or abdominal effusion specimen virtually amounts to a malignant tumor. Preliminary diagnostic criteria were based principally on this assumption.

In studying imprints of 10 control patients without malignancy the assumption was confirmed that not more than 5 per cent of nuclei would be found in the 4k class, even in strongly irritated cases. Nevertheless, in order to remain on the safe side, the lower limit for malignancy was fixed at 10 per cent of 4k nuclei. Consequently values between 5 and 10 per cent had to be considered as suspicious.

An increased number of DNA levels in the 3k class indicates benign irritated pleura or peritoneum, or tumor with diploid karyotype and low rate of mitosis. It is virtually impossible to give an estimate of percentages of 3k values that can be utilized as an index of malignancy. It was therefore decided to bypass this problem, in the hope that all cases of malignant fluids would contain, next to high 3k percentages, large numbers of 4k (or higher polyploid) nuclei, too. In fact, a high incidence (20% and more) of 3k values was observed as an isolated phenomenon in three fluids, which were classified cytophotometrically as "suspicious". In the follow-up one of these turned out to be of patient with benign pleuritis.

No proven false positive cytophotometric diagnosis was made and only three proven false negatives, two of which were found positive by the cytopathologist. The cytophotometric results in proven malignant cases were somewhat better than those obtained with conventional Papanicolaou cytology. This may be partly attributable to the authors' personal interest, which prompted meticulous cell-by cell examination and personally attended sampling. The cytopathologist could afford to spend only a fraction of this time and effort and he was dependent on sampling by lower qualified technicians.

Another advantage of the cytophotometric method or more precisely the staining technique over conventional cytology is that Feulgen-DNA measurements are not upset by the presence of erythrocytes, which are lysed and not stained during the Feulgen procedure, whereas reliable examination of hemorrhagic smears with the Papanicolaou technique may be impossible.

For the cytopathologist minute sample of fluid containing very small number of cells often leads to diagnostic difficulties. Generally cytopathologist will not make diagnosis on single atypical cell. Cytophotometrically however single 8k nucleus definitely indicates malignant cell in our

The following diagnostic criteria, which will be discussed later on, were applied as a working hypothesis

(1) A smear does not contain tumor cells when not more than 5 per cent of the nuclei belong to DNA class 4k or higher and there are no nuclei of class 8k or higher
(2) A smear contains tumor cells when at least 10 per cent of the nuclei are in class 4k or higher or when any undoubtedly single *nucleus has a DNA content of 8k or higher*
(3) A smear with more than 5 per cent but less than 10 per cent of the nuclei in class 4k and 6k should be regarded as suspicious of cancer In addition a smear with a "remarkable percentage of 3k values should be also considered as suspect

After a cytophotometric result of "tumor negative" or "suspicious' slides were screened under the ordinary microscope (in which the slide could be moved more freely) for the presence of large and darkly stained nuclei, which possibly could be classified as 8k nuclei These cells were then measured with the cytophotometer One single 8k nucleus could alter the diagnosis to "tumor-positive This happened in the case of fluids number 8 and 10 (Table III)

The patients were followed up for two to four years. Cases were considered to be proven tumor negative when no tumor was found in the pleura or peritoneum at laparotomy thoracotomy or autopsy or when the patients remained alive at the end of the investigation and had no clinical symptoms of malignancy for at least 18 months The only fluids considered definitely malignant were those in which the presence of tumor cells in the pleura or peritoneum was histologically proven

RESULTS

Table I shows that in a control series of 10 *mesothelial imprints of 10 surgical patients* without malignancy the readings fall completely within the limits postulated for non-malignancy From the 87 specimens examined cytophotometrically 36 were found to be tumor negative (3 of which appeared to be proven false-negative) 43 were tumor-positive (no false-positives) and eight were suspicious. The findings of individual cases are summarized in Tables II to IV Compared with the results of classical cytopathologic examination from 10 cytophotometric tumor-positive fluids histologically proven malignant, three fluids were found cytologically negative, four suspect and three without diagnosis because of various reasons.

The nuclei of lymphocytes were constantly found to show a lower DNA content than meso*thelial or epithelial cells The* ratio mean value diploid mesothelial nuclei to mean value diploid lymphocytic nuclei was generally around 1 6 and varied from 1 2 to 2 6

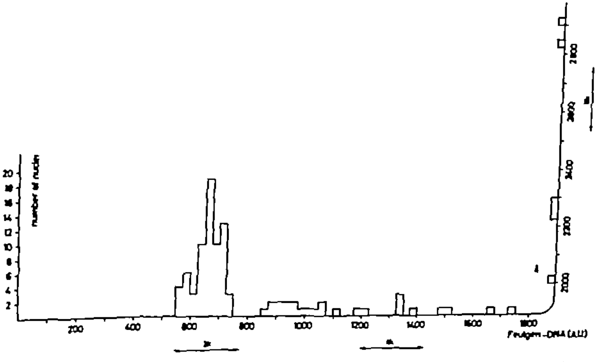

FIG. 3. Patient No. 39 fluid No. 4 (ascites due to ovarian carcinoma) *Mesothelial cells* mean of separated diploid values 662 ± 50 A. U Limits of class 2k 550–775 A. U limits of class 4k 1200–1425 A. U limits of class 8k 2525–2750 A. U *Diagnosis* 14% of mesothelial cells in 4k or higher with % higher than 8k malignancy present.

22	112 P	82	13	5	—	—	5	negative		bronchus c.
23	113 P	94	5	1	—	—	1	negative		bronchus carc.
24	115 P	95	5	—	—	—	—	neg tiv	pleuritis	
25	120 P	100	—	—	—	—	—	neg tiv	pleuritis	
26	124 P	87	13	—	—	—	—	neg tive	L.R.	
	128 P	86	12	2	—	—	2	neg tive		
27	131 P	94	2	2	2	—	4	positive		no autopsy; bronchus rc. (see Table III)
87	44 P	86	10	4	—	—	4	neg tive	p lm embolism	

Inclusive an incidental nucleus < 2k.

† No diagnosis possible due to presence of large quantities of erythrocytes.

material (fluid Nos 8 and 10 Table III) It cannot be overemphasized, however that in such cases there should be no doubt that the nucleus is single.

An important element in presenting the results of cytophotometry is the method of defining the limits of the ploidy classes. This was done by using mesothelial cells as diploid standard and not lymphocytes or granulocytes as is customary [17, ‡] Most workers have found that lymphocytes have Feulgen-DNA content 10 to 20 per cent lower than epithelial cells and a correction factor of 1 1 or 1 2 was therefore used E en artificially swollen lymphocytic nuclei do not reach the Feulgen-DNA readings of mesothelial nuclei Stich *et al* reported the same values for lymphocytes as for intestinal epithelium I our material, however lymphocytes can be ruled out for use as a diploid standard, with or without correction, except for cases of suspected lymphocytic malignancy (Fig 2) This became very clear from the highly variable ratio of Feulgen-DNA values of lymphocytes to those of mesothelial cells in the same smear These differences could possibly be related to differences in chromatin condensation in lymphocytic and mesothelial nuclei, in accordance with the findings of Garcia and Mayall [18] Moreover it is conceivable that lymphocytic and other nuclei show differences in rate of lysis (DNA-loss) during their longer or shorter stay in peritoneal or pleural fluid.

The uthors believe that the results of Sandritter *et al* [14] who examined cervical smears cytophotometrically and found no less than 8 per cent of the hypothetic diagnoses to be false positive, would have been better if these workers had used vaginal cells rather than lymphocytes as diploid standard Unfortunately these authors measured the cellular content of RNA and DNA together by using gallocyamin staining it is impossible, therefore, to make a direct comparison with our results

A study of larger series with higher rate of autopsy may lead to sharper definition of the tentative criteria for malignancy defined in this paper The group "suspicious of tumor (high 5k percentages and 4k percentages between 5 and 10%) could then be reduced to the advantage of the tumor group by lowering the upper limit However this adjustment requires longer follow-up, more autopsies and larger number of specimens

An important disadvantage of the cytophotometric method is, that it is time-consuming and demands complicated apparatus For screening, measuring and calculating one case 1 to 1½ hour is needed, even with the Deeley apparatus, which needs only three seconds for single measurement However when nucleus is found with

TABLE II Cytophotometrically Tumor Negative Fluid

Patient no.	Fluid no. P(leura) A(scites)	Percentage nuclei in DNA class 2c	4c	8c	16c	>16c	≥8c	Pap. staining cytology	Malignancy of pleura or peritoneum proven positive	proven negative	not proven + clinical diagnosis
1	3 P	94	6	—	—	—	—	negative		pericarditis	—
2	5 P	100	—	—				negative		pleuritis	
	14 P	100	—	—		—		negative			
3	7 P	91	7	1	1		2	suspect		I E	
4	9 P	97	3	—	—	—		suspect			no autopsy bronch. carc.
5	11 A	98	2		—	—		negative		heart failure	
6	12 P	94	5	1	—	—	—	[illegible]		hematothorax	
7	26 P	100	—			—	—	negative		pleuritis	
8	31 P	96	2	2	—	—	2	negative		pleuritis	
	33 P	84	16	—	—	—	—	negative			
9	59 P	94	6	—			—			pulm. embolism	
10	73 P	98	2				—	suspect		pleuritis	
11	84 A	88	8	1		—	1	negative		heart failure	
12	87 P	90	6	4		—	4	suspect	autopsy: reticulosarcoma see table IV		
	89 P	92	8	—	—	—		negative			
13	91 P	94	6		—	—		negative	autopsy: breast cancer		
14	92 P	91	7	2	—			negative			
15	93 P	90	6	4			4	positive		pneumonia	
16	95 A	98	2		—	—		negative			breast cancer 1135
	104 A	92	6	2	—		2	negative		autopsy colitis ulcerosa	
	108 A	100	—		—		—	negative			
17	96 P	96	4		2		2	negative			
18	99 P	94	4	2	—		2	negative		embolism	
	102 P	94	2	4	—	—	4	negative			mamma ablata autopsy
	103 P	86	14	—	—	—	—	—			
19	105 P	98	2	—	—	—	—	negative			1963 colon carc. bronchus carc.
20	107 P	100	—	—	—	—	—	negative			
21	111 P	93	7		—	—	—	—		pleuritis	

22	112 P	82	13	5	—	—	5	neg ve		bronchus carc.
23	113 P	94	5	1	—	—	1	neg tive		bronchus carc.
24	115 P	95	5	—	—	—	—	negative	pleuritis	
25	120 P	100		—	—	—		negativ	pleuritis	
26	124 P	87	13	—	—	—		negativa	L E	
	128 P	86	12	2	—	—	2	negative		no autopsy bronchus carc. (see Table III)
27	131 P	34	2	2	2	—	4	positive		
67	44 P	86	10	4	—	—	4	neg tive	p lm. embolism	

Inclusive an incidental nucleus < 2c

† N diagnosis possible due to presence of larg quantities of erythrocytes

material (fluid Nos. 8 and 10, Table III). It cannot be overemphasized, however, that in such cases there should be no doubt that the nucleus is single.

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A study of a larger series with higher rate of autopsy may lead to sharper definition of the tentative criteria for malignancy defined in this paper. The group "suspicious of tumor" (high 5c percentages and 4c percentages between 5 and 10%) could then be reduced to the advantage of the tumor group by lowering the upper limit. However this adjustment requires longer follow up, more autopsies and a larger number of specimens.

An important disadvantage of the cytophotometric method is, that it is time-consuming and demands complicated apparatus. For screening, measuring and calculating one case, 1 to 1½ hour is needed even with the Deeley apparatus, which needs only three seconds for a single measurement. However when nucleus is found with

TABLE III *Cytophotometrically Tumor Positive Fluid*

Patient no.	Fluid no. P(leura) A(scites)	Percentage nuclei in DNA class						Papanicolaou cytology	Malignancy of pleura or peritoneum		
		2k	3k	4k	6k	≥8k	>4k		proven negative	proven positive	not proven + clin. diagnos.
28	4 P	71	8	7	11	—	18	—			no autopsy breast cancer
29	6 P	25	8	47	13	6	66	positive			no autopsy lung cancer
30	8 P	85	3	—	3	5	8	0†		autopsy metast. lung cancer	
31	10 P	84	3	7	3	3	13	0			no autopsy lung cancer
	19 P	63	10	21	3	—	27	negative			
32	13 P	53	10	21	11	3	35	suspect		autopsy metast. mesothelioma	
	58 P	78	6	10	6	—	16	suspect			
	70 P	7	7	21	2	—	26	negative			
	110 A	68	18	14	—	—	14	negative			
	121 A	86	3	11	—	—	11	negative			
33	16 P	72	7	17	4	—	21	pos.		autopsy pleural mesothelioma	
34	20 P	71	12	15		—	17	positive			no autopsy
35	22 P	46	33	17	4	—	21	—		lymph. leucaemia	
	25 P	86	4	10	—	—	10	negative			
36	27 P	50	20	16	14	—	30	positive		autopsy metast. lung cancer	
37	38 P	58	33	13	15	1	29	—			no autopsy
38	40 P	32	28	4	22	14	40	—			no autopsy
39	41 P	64	12	6	8		20	positive		biopsy metast. ovarian carc.	
	42 P	6	17	4	8	2	14	—			
	46 P	50	6	12	18	14	44	—			
	51 P	50	20	5	19	6	30	positive			
	61 P	40	15	23	13	9	45	positive			
	78 P	54	22	10	10	4	24	positive			
	82 A	30	3	47	10	10	67	positive			
	119 A	30	9	20	20	21	61	positive			
40	43 P	42	43	15		—	15	—			
41	49 P	65	10	25			25	suspect		autopsy lymphosarcoma	
	50 P	70	17	4			13	suspect			
42	63 A	56	14	14	10	6	30	positive		biopsy metast. ovarian carcinoma	
43	72 P	63	20	1	2		17	positive			no autopsy lung cancer
44	74 P	62	14	8	9	7	24	positive			no autopsy
45	79 P	20	15	20	33	10	63	positive		biopsy carcinoma	
	80 P	58	21	10	17	14	14	positive			
	85 P	50	3	7	29	11	47				
46	81 P	63	21	12	2	2	16	positive			no autopsy
47	86 P	77	12	11	—	—	11	positive		autopsy metast. ovarian carcinoma	
48	98 P	29	41	16	14	—	30	positive			no autopsy lung cancer
49	101 P	79	7	11		1	14				no autopsy reticulosarcoma
50	122 P	89		4	6	1	11	positive			no autopsy lung cancer
51	125 P	2	28	14	42	14	70	pos.		autopsy metast. ovarian carcinoma	
	129 P	2	18	4	40	36	80	—			
27	126 P	68	12	16	4		20	suspect			no autopsy lung cancer
52	130 P	4	48	10	38		52	positive			no autopsy

* Inclusive an incidental nucleus < 2k

† No diagnosis possible due to paucity of cells

‡ No diagnosis possible due to presence of large quantities of erythrocytes.

an evident 8k value or more further screening can be stopped as this means an absolute criterion for malignancy. Because of the time involved the cytophotometric method in this form will not find a place in routine practice. Its use will be restricted to doubtful cases or when conventional cytologic examination cannot be performed. This only holds as long as adequate automatic screening and integrating apparatuses are not available.

CONCLUSIONS

Tumor diagnosis in pleural and ascitic fluids, based on DNA measurements and using an in-

TABLE IV *Cytophotometrically Tumor-Suspected Fluid*

Patient no	Fluid no P(leura)	Percentage of nuclei in DNA-class						Papanicolaou cytology	Malignancy of pleura		
		2k	3k	4k	6k	> 8k	> 4k		proven negative	proven positive	not proven + clin. diagnosis
41	56 P	86	6		1		8	suspect		autopsy lymphosarcoma	
32	60 P	92	2	6	—	—	6	—		autopsy mesothelioma	
12	94 P	85	5	5	5	—	10†	negative		autopsy malignant reticulosis	
53	97 P	78	20	2	—	—	2	negative			metastasized lung cancer
	97b P	62	36	2	—	—	2				
54	106 P	88	6	6	—	—	6	negative			no autopsy metastasized lung cancer
8	114 P	61	24	5	5	5	15‡	—	pleuritis (see table II)		
55	118 P	84	8	8	—	—	8	negative			mesothelioma?
56	127 P	78	17	3	2	—	5	negative			no autopsy lung cancer

Inclusive an incidental nucleus < 2k.

† Only 70 nuclei were measured, this makes the result of 10% nuclei in class 4k and higher not accurate enough to diagnose the fluid as malignant.

‡ The measured cells in class 6k and 8k were *probably* multinucleated, thus it is not allowed to diagnose the fluid as malignant. It is therefore classified "suspect"

tegrating scanning cytophotometer and standardized Feulgen stained smears is a reliable method, which can give results as accurate as those of the conventional Papanicolaou method. These results can be achieved because of the absence of polyploidy in non-malignant mesothelial cells and lymphocytes. In classifying nuclear DNA values in a smear it was found that mesothelial cells present in the same smear could be used as a reliable diploid standard. Lymphocytes, used by most authors for this purpose, appear to be unsuitable as they show unpredictable differences in Feulgen-DNA quantity ratio with mesothelial nuclei. However, in cases suspected of malignant lymphoreticulosis, lymphocytes have proved to be the only useful standard. Because of its time-consuming nature however the cytophotometric examination will find only a very limited application in routine practice.

SUMMARY

A method is described for diagnosing malignancy in cytologic smears of pleural and ascitic fluid by measurement of the Feulgen-DNA content of nuclei by scanning cytophotometry. The presence of a high incidence of tetraploid and/or higher values have been used as criteria for malignancy. The results obtained with the application of these criteria appeared to be as reliable as those obtained with conventional Papanicolaou cytology. The technique appeared to be too time-consuming to be useful as an alternative method in routine practice.

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Ultrasonic Dispersal of Cervical Cell Aggregates

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A major obstacle to the automation of mass cervical screening has been the absence of satisfactory technique for the dispersal of cell aggregates Methods of separating mammalian tissues into individual cells using enzymatic digestion with trypsin, collagenase,[10] chelating agents, such as E D T A and turbulent shear in laboratory homogenisers[4] have all yielded only low percentage of intact morphologically undamaged single cells We have been unable to trace any reference to technique which will adequately separate aggregates of cervical cells without causing significant morphologic damage We have therefore investigated the feasibility of separating human cervical cell aggregates using novel ultrasonic device This device (nicknamed "Welsh Harp) has already been used successfully to disperse aggregates of bacterial cells[11,12] and certain mammalian cells grown in tissue culture[7] without significant morphologic damage or decrease in viability

MATERIALS AND METHODS

All specimens were collected from women attending regular cervical cytology clinics After the routine cervical scrape for Papanicolaou smears, a second cervical scrape was taken using plastic spatula and the material obtained suspended in 5 ml of deionised water stored overnight at room temperature centrifuged and resuspended in 1 ml of deionised water A small part of this was examined microscopically to determine the state of aggregation of the untreated material The remainder was exposed to sonication under various controlled conditions at room temperature

The sonicator‡ consists of resonant length (1 69 cm) of thin steel wire (diameter 230 μm) clamped at right angles to the tip of a horizontal stainless steel velocity transformer so that the wire is suspended vertically (Fig 1) When the velocity transformer is made to vibrate at 20 kHz flexural or transverse standing wave system is produced along the wire Submerging the terminal portion of the wire in liquid sets up small scale (d) acoustic microstreaming around the transversely oscillating portions[9] with velocity gradient proportional to the square of the displacement amplitude of the wire The depth of immersion is adjusted so that the liquid meniscus meets the wire at displacement node, to prevent the formation of an aerosol

A concentrated cell suspension (0 5 ml) was sonicated in small glass test tube (internal dimensions 0 5 cm diameter by 0 7 cm length) for various times at a series of wire displacement amplitudes Aliquots were removed at intervals and placed in Fuchs-Rosenthal hemocytometer and also on plain glass slide Each cell or cell aggregate in the hemocytometer was counted as one cell unit and the total number of cell units determined In addition the percentage of cells in the form of aggregates was estimated The samples on the slides were spread as thin layer fixed with carbowax/alcohol spray stained by the Papanicolaou method and examined microscopically to detect morphologic damage and also the proportion of cells in the form of aggregates.

RESULTS

Presonication Examination of the untreated cell suspensions revealed that the *degree of cell aggregation* varied greatly from patient to patient, though practically 100 per cent of samples contained aggregates This variation is demonstrated in the histogram (Fig 2) The *degree of aggregation* was found to be independent of cell type

Postsonication The results presented in Figure 3 show the effect of time of sonication in a typical cell sample, at an arbitrary displacement amplitude of 46 μm, on the number of individual cell units and on the percentage of the total cell population in the form of aggregates of two or more, as determined from the hemocytometer readings.

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‡ For further information on the ultrasonic device contact A. R Williams Present address Department of Anatomy Manchester University Oxford Road, Manchester M 13.

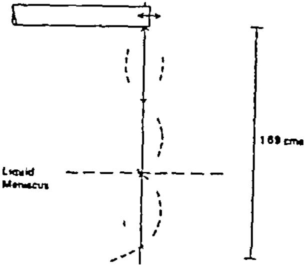

FIG. 1 Diagrammatic representation of the ultrasonic device.

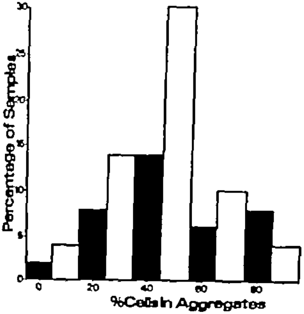

FIG. 2. Patient-to-patient variability. The percentage cell aggregations prior to sonication. (Mean = 47.4% Standard Deviation = 1 0%)

All the samples containing aggregates showed a similar pattern in that the percentage of cells in aggregated form decreased rapidly over the first few minutes to reach a limiting value of from 0 to 5 per cent. There was a corresponding increase in the number of single cells which reached a maximum within five minutes (Fig 3). This was confirmed by the Papanicolaou slide findings. All cell types behaved in a similar manner including abnormal cells.

Detailed microscopic examination of the prepared slides showed that there was no detectable morphologic difference between control cells (Fig 4a) and cells which had been sonicated for five minutes at a displacement amplitude of 46 µm (Fig 4b). To date, most cell separation experiments have been performed with normal cell samples. However two samples which contained abnormal cells were examined after sonication and did not appear to have suffered morphologic damage during separation.

Prolonged sonication at a displacement amplitude of 46 µm did lead to a gradual decrease in the number of cell units and also resulted in the production of a number of morphologically altered cells. Higher displacement amplitudes caused the appearance of enucleated cells and cytoplasmic fragments after shorter treatment times while sonication at a displacement amplitude of 23 µm did not give satisfactory cell separation or any morphologically abnormal cells after treatment times in excess of 20 minutes.

Preliminary experiments have shown that limited predigestion of aggregated cell samples with trypsin or hyaluronidase did not significantly alter the general dispersion pattern described above or increase the efficiency of cell separation. However extensive predigestion with trypsin gave fragile cells readily disrupted by hydrodynamic forces which did not damage control cells. Similarly glutaraldehyde (3%) and sodium metabisulphite (1%) sensitized cells to mechanical shear without appreciably improving dispersion. Chelating agents, such as E.D.T.A. had no measurable effect on either cell fragility or separation.

DISCUSSION

The above results demonstrate that aggregates of human cervical cells in deionised water can be

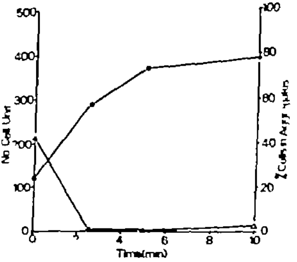

FIG. 3. Effect of time of sonication on human cervical cells at a displacement amplitude of 46 µm ● number of cell units △ percentage of cells in aggregates.

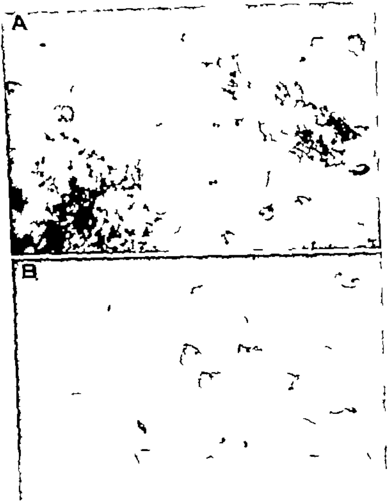

FIG. 4. Human cervical epithelial cells (a) before sonication (b) after sonication.

rapidly dispersed to give suspension consisting predominantly (95–100%) of single cells, the remainder being in the form of two- or three-celled aggregates. The separated cells appear to retain all the morphologic features used to distinguish abnormal cells from their counterparts and the separation is accomplished without a significant difference in the number of cells present ($P > 0.3$). This means that the main obstacle preventing the rapid automation of mass cervical screening, i the presence of large cell aggregates, has been removed.

The mechanism of cell separation and the various ways in which cervical cells respond to the complex hydrodynamic shear field developed around the oscillating wire are not yet fully understood. Both cell disruption and cell separation phenomena are dependent upon the displacement amplitude of the wire and the number of times given cell or cell aggregate passes through the region of maximum velocity gradient. In addition to the hydrodynamic shear forces it is likely that the unusual geometry of the sonication system assists cell separation by accelerating different portions of the same cell aggregates at different times.

The hydrodynamic forces required to disrupt or damage given cell depend largely on the cell's physical condition, which is determined by factors such as the local hormonal and pH levels, the presence of any infection and the general state of health of the patient. Nevertheless, the forces underlying cell aggregation were found to be weaker than the forces maintaining an intact cell membrane in all the samples of cervical cells investigated. Since the samples are dispersed by hydrodynamic forces, it follows that the efficiency of dispersion depends on the displacement amplitude of the wire and the time of exposure but is virtually independent of the number of cells present in suspension

There is no general agreement about the nature of the forces holding cervical cells together to form an aggregate. Rinaldini[11] has suggested that some mammalian cells are held together by ionic bonds involving calcium and magnesium Koss[5] offers electron-microscopic evidence that the parabasal and intermediate cells of the cervical epithelium are linked by desmosomes whereas the superficial cells and malignant cells are not. The nature of the substance which holds cells together at a desmosomal junction (macula adherens) is not known.[1] Ham[2] has stressed the importance of hyaluronic acid and collagen in cell adhesion If such covalent linkages do occur pretreatment of cell aggregates with a suitable enzyme should enable the cells to be mechanically separated more easily Our preliminary experiments have shown that mild predigestion with trypsin and hyaluronidase did not significantly alter the rate or efficiency of the cell separation process

The observation that up to 5 per cent of the total cell population occasionally persists in the form of two- or three-cell aggregates, even after five minutes sonication at a displacement amplitude of 46 μm (Fig 3) indicates the need for certain modifications in the separation procedure These developments are being undertaken and the results will be the subject of a future communication.

SUMMARY

Aggregates of human cervical cells in deionised water were rapidly dispersed by a novel ultrasonic device to give a suspension consisting predominantly of single cells The separated cells appear to retain all the morphologic features used to distinguish abnormal cells from their normal counterparts and the separation is accomplished without a significant decrease in the number of cells present.

ACKNOWLEDGMENTS

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We would like to thank Miss Anne Gregory for technical assistance—Mr G Haddock and Mrs Janet Poole for photography Dr Mary Patricia Jenkins for clinical assistance Cervical Cytology for clinical facilities and Miss Dorothy Simms for typing the manuscript.

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A Comparative Study of the Estrogenic Potential of Two Synthetic Estrogens (Mestranol and Ethinylestradiol)

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Vaginal cytology hormonally induced withdrawal bleeding, endometrial stimulation, cervical mucus ferning etc suffer from discrepancies in estimating the effectiveness of natural and synthetic estrogens and it is difficult to establish which procedure is the most accurate[13,14]

Vaginal cytology has been widely employed in hormonal evaluation as the vaginal epithelium n sex hormone responsive target organ. The vaginal smear result should be expressed in a clear concise and practical way For critical assessment, it is necessary to analyse objectively and express the results accurately Properly chosen indices are able to convey clearly large amount of information.

The index chosen should be obtained from differential cell count—including all layers of the squamous epithelium. We believe that the Superficial Cell Index (SCI) fulfils the above criterion, but the Karyopiknotic Index (KPI) and the Maturation Index (MI) are also valuable guides

The estrogenicity of a compound not only depends upon the dose but also upon the patient hormonal status and the responsiveness of their reproductive organs Patients with endogenous estrogen or progestogen production or those receiving more than one exogenous hormone cannot be used for such study because of the synergistic and antagonistic effects of these two hormones

The young ovariectomized woman with an intact uterus or the postmenopausal women with sensitive target sex organ is suitable for such study Close control of these cases allows an assessment of the uniformity of the response of the vaginal and endometrial epithelium[14]

There is lack of precise information on the real estrogenicity of various natural and synthetic estrogens which are used in hormonal therapy As we had access to a large number of suitable women as defined above, we saw an excellent opportunity for such a trial

Initially we decided to (1) establish the relative estrogenicity of two synthetic compounds, ethinylestradiol and mestranol and (2) establish 0.05 and 0 1 mg of mestranol as standard reference preparations for the comparative study of other compounds.

MATERIAL AND METHODS

The patients were selected from a large group with primary amenorrhea (e g gonadal dysgenesis, hypothalamic amenorrhea, etc.) secondary amenorrhea (e.g. premature ovarian failure, young surgical castrates, hypopituitarism, etc.) and postmenopausal women. All these women required hormonal replacement therapy and at the same time were suitable for inclusion in this trial

Patients with chromosomal abnormalities (Turner Syndrome, etc.) or those with diminished or abnormal response to the administered estrogen were excluded from the study

Vaginal cytology was used as the main method for the assessment of the response and all patients had vaginal smear prior to commencing the estrogen therapy All had either an atrophic pattern (MI = 50-50-0) or hypotrophic (MI = 5-85-0) with mean value of 10-87 3 in patients with primary amenorrhoea and 18-79-3 in the other two groups.

Those patients with proliferative intermediate cell pattern (e g. MI = 0–95–5) and those postmenopausal women who were judged to have continuing endogenous estrogen activity (MI = 0–80–20) were omitted

Dosage

0 025, 0 05 and 0 1 mg of ethinylestradiol or mestranol were given orally for period of ten days. Frequent vaginal smears were performed during this period.

In some women, after a few cycles, the cell patterns exhibited extremely marked cytolysis of the glycogen-containing intermediate cells and low percentage of the karyopyknotic superficial cells These patients were also excluded as these changes in the vaginal cytology made comparison with other groups impossible.

TABLE I. *Effect of Mestranol as Determined by the Vaginal Cytology in 40 Women. Estrogenic Effect of 10 Days' Course of Treatment. The Maturation and Superficial Cell Index Expressed in Mean Values*

	Mestranol		
	0.02 mg	0.05 mg	0.10 mg
Number of cases (n)	[illegible]	25	14
Mean Maturation Index			
before	10-87-3	15-82-3	3-94-3
after	1-87-12	0-67-33	0-43-57
Mean diff. in Superficial Cell Index ± S.D.	10.4 ± 8.[illegible]	50.0 ± 18.5	54.1 ± 26.7

40 women were finally included in the trial:

Pure gonadal dysgenesis	[illegible]
Premature ovarian failure	7
Young surgical castrates	3
Postmenopausal women (age range 40-50 yrs)	[illegible]
Total	40

The 40 patients were observed following the ten days of estrogen therapy and in those cases showing delayed regression of estrogenic effects, chlormadinone acetate 4 mg daily was given for ten days. A reduction of the superficial cell percentage accompanied by a shift to the left in the Maturation Index was observed in all these patients.

e.g. 0-40-60 prior to chlormadinone acetate
0-95-5 after ten days chlormadinone acetate 4 mg/day

A few women had an insignificant reduction in the superficial cell percentage.

RESULTS

A significant change in the cell population of the vaginal epithelium was noticed after ten days of mestranol or ethinylestradiol. As can be seen in Table I, a progressive increase in the Superficial Cell Index is apparent. (See also Fig. 1)

A distinct decrease in the number of parabasal and an increase in the superficial cell numbers compared with the pre-treatment state can be seen. Changes in the percentage of the intermediate type of cells were not significant (e.g. mestranol 0.02 mg: MI = 10-87-3 before and MI = 1-87-12 after therapy). Sometimes there were, however, no changes at all (Table I).

The effects of estrogen on the vaginal epithelium in all 40 women after ten days therapy were quite uniform. In most women a 4 mg dose of chlormadinone acetate given for ten days caused a definite and rapid regression of the estrogenic effects. The number of superficial cells decreased and the small intermediate cells became predominant, to be later replaced by the oval parabasal cells. The regression was considered to be complete when the smear contained mainly small intermediate cells and parabasal cells with numerous leukocytes and some necrotic debris.

The Superficial Cell Index was chosen to measure the estrogenic effect on the vaginal epithelium. However, we were not interested in evaluating individual responses but in obtaining *best estimates of the mean effect of two different estrogens in women as a bioassay*. Neither of the commonly accepted estrogenicity indices meet the requirements for a good bioassay procedure (linear response to the log dose and a stable variance, i.e. independence of the dispersion of individual results from the dose). Apart from the

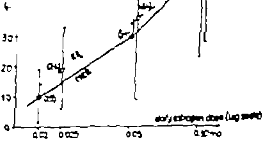

FIG. 1. a) Comparison of effects of two estrogens (mestranol (MEE) and ethinylestradiol (EE)) on the vaginal epithelium response in amenorrheic women. b) The relationship between the Superficial Cell Index (SCI) and the dose of estrogen administered orally for 10 days.

TABLE II. *Estrogenicity of Ethinylestradiol as Determined by the Vaginal Cytology in 40 Women Estrogenic Effect of 10 Day Therapy*

	Ethinylestradiol		
	0 025 mg	05 mg	10 mg
Number of cases ( )	25	15	12
Mean Maturation Index			
before	18–79–3	2–95–3	21 77–2
after	1 77–22	0–64–36	1–47 52
Mean diff in Superficial Cell Index ± S D	19 2 ± 13 3	36 2 ± 27 4	50 0 ± 28 1

Superficial Cell Index was therefore used the Dynamic Estrogenicity Index (DEI) developed for the sole purpose of bioassay of estrogen preparations in women All the mean values are collected in Table I and the comparison of effects of the two estrogen studies are shown in Figure 1

DISCUSSION

Using the Superficial Cell Index, both estrogen studies proved to be indistinguishable in the response of the vaginal epithelium compared to the pretreatment state (Table I) Therefore they may be interchangeable as reference compounds in studying the estrogenicity of different preparations.

Tablets of 0 05 and 0 1 mg mestranol were chosen for further studies. The increase in the Superficial Cell Index to 30 0 (SD ± 18 5) following treatment *with mestranol 0.05 mg and* the increase to 54.1 (SD ± 26.7) following mestranol 0.1 mg have been adopted as standard vaginal responses

If the term estrogenic effect" used in vaginal cytology is taken to mean the local vaginal effect induced by estrogens, this terminology can be used and accepted for practical purposes only in the evaluation of the estrogenic effect of individual preparations Only in these situations does there exist direct and proportional relationship between the vaginal cytology and the estrogen dosage

Such quantitative evaluation may only be achieved in patients with atrophic vaginal smears Determining the duration of estrogenic effect and the return to the pretreatment state with the reappearance of the atrophic changes in the vaginal epithelium, constitutes particular problem The fact that the indices may remain elevated for some time, even up to ten days after estrogen therapy had been discontinued, shows the limitation of exfoliative cytology in the assessment of patients undergoing cyclical hormone therapy

SUMMARY

I clinical practice, there is an apparent lack of precise information on the estrogenicity of various synthetic and natural estrogens, which are used for hormonal treatment. Only their ovulation suppression properties have been relatively well explored It was decided that further studies would be based on vaginal cytology

One of us (J T) collected large group of patients with primary and secondary amenorrhea All these patients required hormonal replacement therapy and appeared adequate for trials to determine the estrogenicity of various synthetic estrogen compounds. The largest group consisted of postmenopausal women

A uniform technique of medication with various doses of mestranol and ethinylestradiol for ten day periods is presented as a method for the assessment of their estrogenic potency Vaginal cytology was performed in 40 carefully selected amenorrheic and postmenopausal women with highly sensitive vaginal epithelium. In the course of these investigations the patients with vaginal cytolytic cytology and diminished responsiveness to estrogen therapy were excluded from the study as well as several older women with diminished or abnormal responses.

The induced estrogenic effects on the vaginal epithelium were measured by the shift of the Maturation Index to the *right* and *an increase* in the Superficial Cell Index. In most patients definite and rapid regression was obtained by the administration of chlormadinone acetate 4 mg daily for ten days

The increase in the Superficial Cell Index in comparison to the pretreatment state was 30 0 (SD ± 18 5) for mestranol 0 05 mg and 54 1 (SD ± 26.7) for mestranol 0.1 mg. This may be adopted as *standard reference* vaginal response in further comparative studies of other estrogenic compounds. The vaginal response to 0 05 mg daily of mestranol did not differ significantly from that of 0 05 mg of ethinylestradiol.

Further comparative studies are necessary between 0 02 mg mestranol and ethinylestradiol and 0 025 mg of ethinylestradiol and mestranol.

TABLE I. Ef i gency f Mestranol Daily Dose ... the Vaginal Cytology in 40 Women. Estrogen Effect ... 10 Days C ... f Treatment. The Maturation and Superficial Cell Indices are Expressed in Mean ...

	Mestranol		
	0.02 mg	0.05 mg	0.10 mg
Number of cases (n)	9	25	14
Mean Maturation Index			
before	10-87-3	15-82-3	3-94-3
after	1-87-12	0-67-33	0-43-57
Mean diff. in Superficial Cell Index ± S.D.	10.4 ± 8.[illegible]	30.0 ± 18.5	54.1 ± [illegible]7

40 women were finally included in the trial:

Pure gonadal dysgenesis	1
Premature ovarian failure	7
Young surgical castrates	3
Postmenopausal women (age range 48-59 yrs)	29
Total	40

The 40 patients were observed following the ten days of estrogen therapy and in those cases showing delayed regression of estrogenic effects, chlormadinone acetate 4 mg daily was given for ten days. A reduction of the superficial cell percentage accompanied by a shift to the left in the Maturation Index was observed in all these patients.

e.g. 0-40-60 prior to chlormadinone acetate
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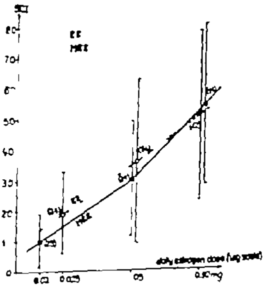

FIG. 1. a) Comparison of effects of two estrogens (mestranol (MEE) and ethinylestradiol (EE)) on the vaginal epithelium response in amenorrheic women. b) The relationship between the Superficial Cell Index (SCI) and the dose of estrogen administered orally for 10 days.

TABLE 1B *Estrogenicity of Ethinylestradiol as Determined by the Vaginal Cytology in 40 Women. Estrogenic Effect of 10 Days Therapy*

	Ethinylestradiol		
	0.02 mg	0.05 mg	0.10 mg
Number of cases ( )	25	15	12
Mean Maturation Index			
before	18-79-3	2-95-3	21 77 2
after	1 77 22	0-64-36	1-47-52
Mean diff. in Superficial Cell Index ± S.D	10.2 ± 13 3	36 2 ± 27 4	50 0 ± 28 1

Superficial Cell Index we therefore used the Dynamic Estrogenicity Index (DEI) developed for the sole purpose of bioassay of estrogen preparations in women All the mean values are collected in Table I and the comparison of effects of the two estrogen studies are shown in Figure 1

DISCUSSION

Using the Superficial Cell Index, both estrogen studies proved to be indistinguishable in the response of the vaginal epithelium compared to the pretreatment state (Table I) Therefore they may be interchangeable as reference compounds in studying the estrogenicity of different preparations

Tablets of 0 05 and 0 1 mg mestranol were chosen for further studies. The increase in the Superficial Cell Index to 30 0 (SD ± 18 5) following treatment with mestranol 0 05 mg and the increase to 54 1 (SD ± 26.7) following mestranol 0 1 mg have been adopted as standard vaginal responses.

If the term estrogenic effect" used in vaginal cytology is taken to mean the local vaginal effect induced by estrogens, this terminology can be used and accepted for practical purposes only in the evaluation of the estrogenic effect of individual preparations Only in these situations does there exist direct and proportional relationship between the vaginal cytology and the estrogen dosage

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The increase in the Superficial Cell Index in comparison to the pretreatment state was 30 0 (SD ± 18.5) for mestranol 0 05 mg and 54 1 (SD ± 26.7) for mestranol 0.1 mg This may be adopted as a standard reference vaginal response in further comparative studies of other estrogenic compounds The vaginal response to 0 05 mg daily of mestranol did not differ significantly from that of 0 05 mg of ethinylestradiol.

Further comparative studies are necessary between 0 02 mg mestranol and ethinylestradiol and 0 025 mg of ethinylestradiol and mestranol.

TABLE I. Estrogenicity of Mestranol as Determined by the Vaginal Cytology in 40 Women. Estrogenic Effect of 10 Days Course of Treatment. The Maturation and Superficial Cell Index are Expressed in Mean Values

	Mestranol		
	0.02 mg	0.05 mg	0.10 mg
Number of cases (n)	3	23	14
Mean Maturation Index			
before	10-87-3	13-82-3	3-94-3
after	1-87-1	0-67-33	0-43-57
Mean diff. in Superficial Cell Index ± S.D.	10.4 ± 8.7	30.0 ± 18.5	54.1 ± 26.7

40 women were finally included in the trial:

Pure gonadal dysgenesis	[illegible]
Premature ovarian failure	7
Young surgical castrates	5
Postmenopausal women (age range 48-59 yrs)	[illegible]
Total	40

The 40 patients were observed following the ten days of estrogen therapy and in those cases showing delayed regression of estrogenic effects, chlormadinone acetate 4 mg daily was given for ten days. A reduction of the superficial cell percentage accompanied by a shift to the left in the Maturation Index was observed in all these patients.

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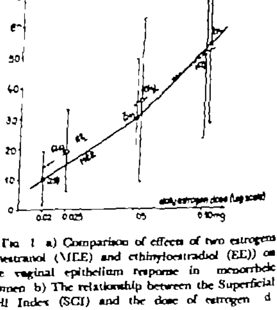

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TABLE III *Estrogenicity of Ethinyl Estradiol as Determined by the Vaginal Cytology in 40 Women Estrogenic Effect of 10 Day Therapy*

	Ethinylestradiol		
	0 02 mg	0 05 mg	10 mg
Number of cases ( )	25	15	12
Mean Maturation Index			
before	18–79–3	2–95–3	21 77–2
after	1 77–22	0–64–36	1–47 52
Mean diff. in Superficial Cell Index ± S.D	19 2 ± 13 3	36 2 ± 27 4	50 0 ± 28 1

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Further comparative studies are necessary between 0 02 mg mestranol and ethinylestradiol and 0 025 mg of ethinylestradiol and mestranol.

TABLE I. *Estrogenic Effect of Mestranol as Determined by the Vaginal Cytology in 40 Women Following 10 Day Course of Treatment. The Maturation and Superficial Cell Index are Expressed as Mean Values*

	Mestranol		
	0.02 mg	0.05 mg	0.10 mg
Number of cases (n)	[illegible]	25	14
Mean Maturation Index			
before	10-87-3	15-82-3	3-94-3
after	1-87-12	0-67-33	0-43-57
Mean diff. in Superficial Cell Index ± S.D.	10.4 ± 8.9	30.0 ± 18.5	54.1 ± 26.7

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A significant change in the cell population of the vaginal epithelium was noticed after ten days of mestranol or ethinylestradiol. As can be seen in Table I a progressive increase in the Superficial Cell Index is apparent (See also Fig 1)

A distinct decrease in the number of parabasal and an increase in the superficial cell numbers compared with the pre-treatment state can be seen. Changes in the percentage of the intermediate type of cell were not significant (e.g. mestranol 0.02 mg MI = 10–87 3 before and MI = 1 87–12 after therapy) Sometimes there were however no changes at all (Table I)

The effects of estrogen on the vaginal epithelium in all 40 women after ten days therapy were quite uniform. In most women a 4 mg dose of chlormadinone acetate given for ten days caused a definite and rapid regression of the estrogenic effect. The number of superficial cells decreased and the small intermediate cell became predominant, to be later replaced by the oval parabasal cells. The regression was considered to be complete when the smear contained mainly small intermediate cells and parabasal cells with numerous leukocytes and some necrotic debris.

The Superficial Cell Index was chosen to measure the estrogenic effect on the vaginal epithelium. However we were not interested in evaluating individual responses but in obtaining best estimates of the mean effect of two different estrogens in women as a bioassay. Neither of the commonly accepted estrogenicity indices meet the requirements for a good bioassay procedure (linear response to the log dose and a stable variance i.e. independence of the dispersion of individual results from the dose) Apart from the

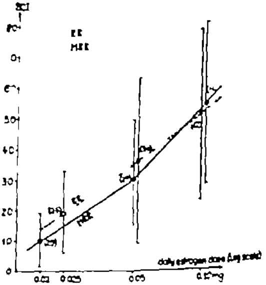

FIG. 1 a) Comparison of effects of two estrogens (mestranol (MEE) and ethinyloestradiol (EE)) on the vaginal epithelium response in amenorrheic women. b) The relationship between the Superficial Cell Index (SCI) and the dose of estrogen administered orally for 10 days

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Preliminary Report on the Incidence of Cervical Carcinoma in Situ in Iraq

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The incidence of carcinoma of the cervix varies very much from one community to another. Since the introduction of mass-screening techniques for cytologic study, these studies have become of definite practical value in trying to reduce the overall incidence of cervical carcinoma in any community.

No such study has so far been carried out in Iraq. The present study was therefore undertaken to find out how the incidence of cervical carcinoma among a predominantly Moslem low income group of patients compares with results in other parts of the world, and to try to detect any factors that may affect this incidence.

MATERIAL AND METHODS

The patients in this study were women who attended the gynecologic outpatient clinic of the Department of Obstetrics and Gynaecology, University of Baghdad. These patients are approximately 98 per cent Moslem, and almost all come from working-class families. A total of 1 152 women, all above the age of twenty and seeking medical advice for various gynecologic complaints, were examined during a six-month period ending in January 1970. Each woman was interviewed as to her medical history, then she was examined clinically. Cervical scrapings and vaginal smears were taken from each patient. The smears were stained with both the Papanicolaou and supravital stains (brilliant cresyl blue 1).

RESULTS

Of the 1 152 patients only four were found to have carcinoma in situ, giving an incidence of 3.4 per thousand.

The age distribution of the patients is given in Table I and their parity in Table II. The average parity is 5.1 children per patient. Table III gives the data concerning the positive cases. In two of these four cases, though cytologic examination was strongly suggestive of carcinoma in situ, the lesion was not found by the punch biopsy and only appeared in the conization specimen taken one week later. No cases of invasive carcinoma were detected in this study.

DISCUSSION

An incidence of 3.4 per thousand smears is one of the lowest in published series. Fiege and Bennington[1] examining 18,000 women in Seattle, reported an incidence of 16 per thousand women in a poor community as opposed to six per thousand in private or semi-private patients in the same district. Way and co-workers[2] found six in situ or microinvasive carcinomas among 670 smears from women attending general outpatient clinics, giving an incidence of about nine per thousand cases. Stern found an incidence of six per thousand in 39 000 smears from healthy women. About half of these positive smears were from invasive carcinomas. Christopherson, Parker and Drye, studying the results of over 100,000 smears, found a rather similar incidence of 6.5 per thousand; of these 2.8 per thousand were invasive carcinomas.

The low incidence in the present series is probably due to the fact that all Moslem husbands are circumcised. Usually circumcision is done between birth and the age of 10 or 12 years. Circumcision is an important factor, as has been shown by many studies, amongst them a very detailed one by Wynder and co-workers. In the present results the effect of circumcision seems to have offset the other factors such as early marriage (mostly at the age of fourteen to twenty). These women also have very large families, as shown by the high average parity of 5.1 children per thousand women. Both of these factors increase the incidence of cervical carcinoma.[4,5] These women also come from working-class families, an important factor in increasing the incidence of carcinoma.

Another important feature of this study is the apparent better reliability of the supravital stain as compared with Papanicolaou stain. Two of the four cases would have been missed had the Papanicolaou stain alone been used. The supravital stain has the advantage of not needing any complicated staining technique. But of course, being a stain of fresh preparations, it can only be used where the slides can be examined immediately

ACKNOWLEDGMENTS

Acknowledgments are due to Mr. A. J. Wilson (G. D. Searle & Co. Ltd., High Wycombe, England) and to Dr. H. Kopera (N. V. Organon, Oss, Holland) for the generous supply of mestranol and ethinylestradiol used in this trial.

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Chromosomal Findings in a Case of Leukemic Reticuloendotheliosis

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Leukemic reticuloendotheliosis is rare malignant disease of the reticuloendothelial system, characterized by extensive proliferation of neoplastic reticulum cells in various organs and tissues, and by the presence of reticulum cells in the peripheral blood The neoplastic reticulum cell can have different aspects, from the ery undifferentiated type, to the monocytoid" type or the "lymphoid" type

The cytogenetic findings obtained in case of leukemic reticuloendotheliosis in its acute variety are presented in this paper

CASE REPORT

B F male, age 43 I May 1968 the patient complained of weakness, fatigue anorexia, evening fever (37.5–37.8°C) and weight loss The patient consulted the family doctor liver and spleen enlargement were noted, and the patient was advised to seek hospital admission

On admission to the Clinica del Lavoro (June 6, 1968), the liver was palpable 6 cm below the right costal margin, the spleen was palpable t the transverse umbilical line there was moderate ascites, no superficial nodes palpable The laboratory findings were RBC sedimentation rate 32 Hb 9.6 g/100 ml, RBC 3,200,000/mm³ WBC 3200/mm³ with neutrophils 75%, eosinophils 1% lymphocytes 14% monocytes 5%, metamyelocytes 5% platelets 210,000/mm³ prothrombin activity 100%, normal bleeding and coagulation times total serum bilirubin 0.50 mg/100 ml, normal blood sugar and urea nitrogen

A bone marrow aspiration (June 19) showed cellular marrow ith myeloblasts 1.2%, promyelocytes 7.8%, myelocytes 15.6%, metamyelocytes 16.2% neutrophils 15% eosinophils 0.4%, basophils 0.2% proerythroblasts 1%, basophilic erythroblasts 9.6%, polychromatophilic erythroblasts 14.8%, orthochromatic erythroblasts 7%, lymphocytes 2.2%, monocytes 0.2%, reticulum cells 8.8% the myeloid-erythroid ratio was 1 2 The reticulum cells were in groups, so that some of them had escaped the initial observation The reticulum cells presented usually an irregular cytoplasmic border delicate basophilic cytoplasm, round or indented nucleus with spongy chromatin and nucleolus

During hospital admission left pleural effusion appeared and an increase of ascites was noted. The patient received only supportive treatment and was discharged on his request on July 6 in poor general conditions

He was readmitted few days later The laboratory findings were RBC sedimentation rate 69 Hb 7.2 g/100 ml, RBC 2,200,000/mm³ WBC 15,500/mmc with neutrophils 49%, eosinophils 4% basophils 1% lymphocytes 16% monocytes 4%, reticulum cells 26% (Fig 1) erythroblasts 2% WBC The reticulum cells were alkaline phosphatase neg tive, while the neutrophil alkaline phosphatase score according to Kaplow was elevated [136]

On July 16 the patient was transferred to surgical department and underwent exploratory laparotomy Hepatosplenomegaly was confirmed and severe involvement of the mesenteric lymph nodes was found A specimen of ascitic fluid taken at operation was reported by us as follows Positive for malignant cells, cytologic pattern of lymphoma, probably reticulum cell sarcoma (Fig 2) The pathologic report on lymph node biopsy was Reticulum cell sarcoma (Fig. 3)

The patient was treated with blood transfusions, cyclophosphamide, and later with sarcolysin During treatment the spleen enlarged progressively and numerous axillary and inguinal lymph nodes became palpable, but the patient showed moderate subjective improvement and was discharged on September 10

He was readmitted again few days later in very poor general conditions. During the third admission he worsened progressively The anemia became more marked On September 20, numerous nodules about 0.5 cm in diameter were observed on the skin of the trunk and of limbs.

The patient died on October 15, 1968. The autopsy findings were mainly hemorrhage prob-

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TABLE I

Age Distribution Among the Series of Patients Examined

Age group	No. of patients	Percentage
20–29	439	38.1%
30–39	407	35.3%
40–49	256	22.2%
50 and over	50	4.4%
Total	115	100.0%

TABLE II

Parity Among the Series of Patients Examined

Parity	No. of Patients
Zero and one	221
Two and three	253
Four and five	208
Over five	470

TABLE III

Details of Positive Cases Detected

Case	Age	Parity	Years married	Cytology		Histopathology	
				Pap. stain	Supravital stain	Punch biopsy	Cone biopsy
1	50	9	33	Positive	Positive	Negative	Carcinoma in situ
2	43	4	23	Positive	Positive	Negative	Carcinoma in situ
3	40	4	24	Negative	Positive	Carcinoma in situ	Not done
4	24	3	11	Negative	Positive	Carcinoma in situ	Not done

after taking the smear which may not always be practical.

SUMMARY

The results of cervical cytology for a presenting sample of women attending a gynecologic clinic in a Moslem working class community are surveyed. Of 1 152 cases, four were positive for carcinoma in situ. A discussion of the cause of the low incidence is offered. Supravital staining of the cervical smear seems to give better results than the Papanicolaou procedure.

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Chromosomal Findings in a Case of Leukemic Reticuloendotheliosis

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Leukemic reticuloendotheliosis is a rare malignant disease of the reticuloendothelial system, characterized by extensive proliferation of neoplastic reticulum cells in various organs and tissues, and by the presence of reticulum cells in the peripheral blood The neoplastic reticulum cell can have different aspects, from the very undifferentiated type, to the monocytoid" type or the "lymphoid" type

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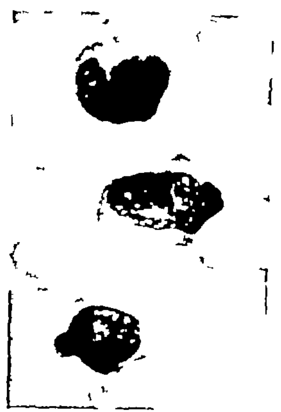

FIG. 1. Neoplastic reticulum cells "monocytoid" type in circulating blood. May Grünwald-Giemsa stain. ×1600.

ably from esophageal varices, diffuse reticulum cell proliferation involving liver, spleen, lymph nodes, and in skin nodules.

MATERIALS AND METHODS

Direct preparations of bone marrow and peripheral blood, and cultures of peripheral blood for chromosome studies were made at the dates reported in Table I. All material for chromosome studies was collected before chemotherapy.

Direct preparations were obtained by incubating bone marrow for 45 hours and peripheral blood for 2 hours with Colcemid® (Ciba), washing the cells twice with Hanks solution, treating with diluted Hanks solution (1 part Hanks, 4 parts distilled water), fixing in methanol acetic acid (3:1); the chromosome spreads were obtained by the air-drying method with slight flaming and were stained by Giemsa solution.

Short term leukocyte cultures were carried out with and without phytohemagglutinin (PHA). Cultures for 48 to 50 hours without PHA were set up by *mixing 1 part of leukocyte-enriched* plasma obtained by spontaneous sedimentation without PHA for 2 hours, and 2 parts of TC Medium 199 Difco. The 72 hour cultures were set up in two *different ways: (1) by a micro*method (3 drops heparinized blood, 9 ml TC *Medium 199 Difco, 1 ml* autologous plasma, 0.2 ml PHA M Difco); (2) by a macromethod, modified from Moorhead et al.[9] (1 part of leukocyte-enriched plasma obtained by spontaneous sedimentation without PHA and 2 parts of TC Medium 199 *Difco were preincubated for 1 hour* at 37°C in a large flat-bottom glass bottle, to remove the cells able to adhere to the glass, i.e. polymorphonuclear cells, monocytes etc.; the supernatant was removed and the cultures were started with PHA). After 70 hours Colcemid was *added for two hours and cells were harvested* according to the method described for the direct preparation.

The direct preparations and the cultures without PHA were carried out with the aim of studying the chromosomes of cells capable of dividing without being stimulated.

RESULTS

The results of the chromosome studies are summarized in Table I. In a direct preparation of bone marrow, *where reticulum cells represented* 8.8 per cent of the cell population, a marker chromosome was detected in direct microscopic observation in 18 out of 100 metaphases and in other metaphases not suitable for counting. The

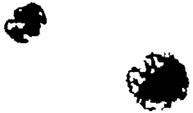

FIG. Reticulum cells in ascitic fluid. Note the indented nucleus and the prominent nucleoli. *Papanicolaou stain. ×1600.*

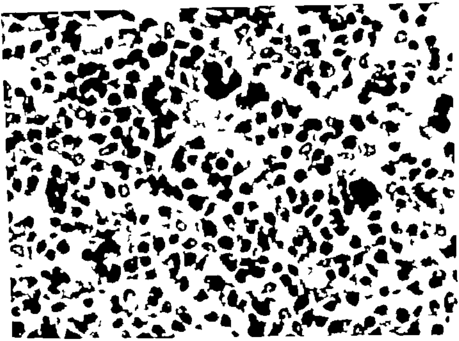

FIG. 3. Section of lymph node. Reticulum cells sarcoma. Hematoxylin and eosin. ×640

TABLE I

Date	Source	Method	Chromosome number				Total counts	Observations
			≤45	≤45 + marker	46	46 + marker		
6/19/68	BM	Direct	29	6	53	12	100	Marker observed in some more mitoses not suitable for counting
6/26/68	PB	Culture 50 h without PHA	—	—	—	—	—	Rare mitoses, not suitable for counting
6/28/68	PB	Culture 72 h with PHA						
		micromethod	3	—	7	1	11	Blast transformation 21.8%
		macromethod	4	—	45	—	49	Blast transformation 31.6%
7/11/68	PB	Direct	—	1	—	—	1	Some more mitoses not suitable for counting
		Culture 48 h without PHA	—	—	—	—	—	No mitoses found

BM bone marrow. PB peripheral blood.

marker was a very minute chromosome without an evident centromere (Fig. 4). The chromosome analysis showed that the karyotype of the abnormal clone was 47,XY,mar+ according to the nomenclature of the Chicago Conference, while that of the cells without marker was a normal male complement, 46,XY. In several metaphases with the marker, one chromosome classified as E18 seemed to be more acrocentric than usual (Fig. 4) but the finding was not consistent. Since the total length of this chromosome was equal to that of the other chromosomes of the pair, a pericentric inversion in an E18 chromosome seemed more probable than a simple short-arm deletion.

A high rate (35%) of hypodiploid cells both with and without the marker chromosome was present. In the hypodiploid cells, chromosomes

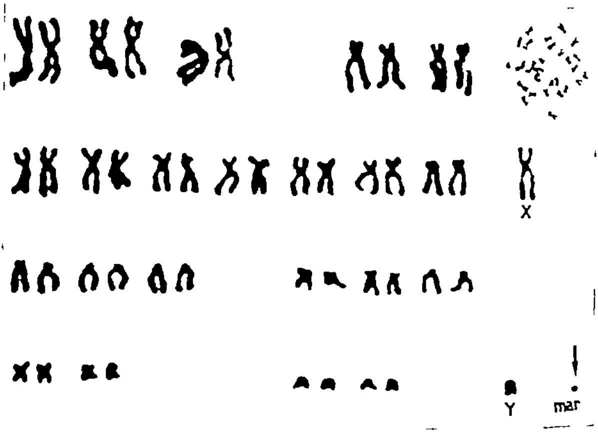

FIG. 4 Spread and karyotype of a metaphase with the minute marker chromosome (arrowed) Note the fuzzy appearance of chromosomes. Bone marrow direct preparation.

were missing from various groups, with random loss. The chromosomes of the abnormal metaphases were sometimes fuzzy (Fig 4) and were often more slender and less contracted than those of the normal metaphases In some metaphases with the abnormal karyotype a basophilic cytoplasmic background with no granules was present which permitted a differentiation from the metaphases occurring in cells of the myeloid series, but not from those occurring in the erythroid series.

No mitoses, or metaphases unsuitable for counting were found in two 48 to 50 hour leukocyte cultures without PHA, set up at two different times when the circulating reticulum cells were 5 and 26 per cent respectively

A normal 46 XY male karyotype was present in the cells obtained in 72 hour cultures with PHA, except that in one cell with the typical abnormal karyotype found in the micromethod preparation

In the 72 hour cultures an impaired response of lymphocytes to PHA was observed In fact the
asts was 21 8 per cent of the mononuclear
e micromethod and 31 6 per cent in
ethod preparation, while in cultures
al subjects in our laboratory under identical experimental conditions, the percentage of blast cells at 72 hours is 75 to 90.

A single hypodiploid cell with the marker chromosome was found in a direct preparation of peripheral blood made at the time when circulating reticulum cells were present (26% of 15,500 WBC) Some more mitoses were found which had such a fuzzy appearance that they could not even be scored for the presence of the marker chromosome

DISCUSSION

The neoplastic reticul m cells observed in bone marrow peripheral blood, ascitic fluid in the case reported are similar to those observed by other authors in cases of leukemic reticuloendotheliosis The clone with the consistent abnormal karyotype in the bone marrow seemed to represent the neoplastic reticulum cell. In fact the patient was untreated at the time of the chromosome study and therefore the chromosome abnormality could not be iatrogenic.

The presence of some mitoses in the direct preparation of peripheral blood and in the 48 hour leukocyte cultures without PHA could be attributed to neoplastic cells capable of dividing without being stimulated unfortunately in these preparations only one cell suitable for analysis was found, and this metaphase possessed the characteristic marker The abnormal karyotype was present also in one cell in the 72 hour culture with PHA (micromethod) The low rate of abnormal mitoses in the preparations from peripheral blood may be due to scant proliferation in vitro of the neoplastic reticulum cell

The origin of the marker chromosome cannot be suggested. The fact that it was transmitted from cell to cell, giving rise to a clone, makes one suppose that it was centric, even though the centromere was not evident

The chromosomal abnormality found in this case of leukemic reticuloendotheliosis is different from those reported in other similar cases,[17] thus confirming once more the pleiomorphic cytogenetic aspect of the acute hemopathies[13] Structural and numerical abnormalities of the E17 18 chromosomes have been reported in several cases of malignant lymphoma[14] in this case, however structural abnormality of an E18 chromosome was not consistent finding

The normal karyotype of most cells in the bone marrow where normal myelo- and erythropoesis was present and the neoplastic reticulum cells represented only 8 8 per cent of the cell population, suggests the independence of the myeloid and erythroid series from the neoplastic clone In the same way the normal male complement in the circulating lymphocytes suggests the independence of the lymphoblastic line from the neoplastic reticulum cell

However the blast transformation of lymphocytes in the presence of PHA seemed to be impaired, in analogy with the observations made in other neoplastic diseases of the reticuloendothelial system This impaired response could not be due to any drug, since the patient was untreated at the time of the culture studies these data are at variance from those obtained in the case of Yam et al[17] where the impaired response was attributable to the steroid treatment No data are available in our case to suggest whether the scant stimulation of lymphocytes by PHA might have been due to functional impairment of these cells or to some plasmatic factor inhibiting transformation of normal lymphocytes

SUMMARY

Chromosome studies were carried out on cells from bone marrow and peripheral blood in patient with leukemic reticuloendotheliosis. I direct preparation of bone marrow an abnormal clone with marker chromosome was detected this clone represented 18 per cent of the metaphases studied. The karyotype of the abnormal clone was 47 XY mar+ where the marker was a minute chromosome I some metaphases a structural abnormality of an E18 chromosome was suspected Some cells with the marker were hypodiploid, with missing chromosomes from various groups Chromosome counts and analysis performed on the remaining bone marrow metaphases, as well as on peripheral blood lymphocytes cultured for 72 hours with phytohemagglutinin (PHA) showed normal complement (46 XY) However an impaired response of lymphocytes to PHA was noted One metaphase with the marker chromosome was observed in a direct preparation of peripheral blood obtained at time when circul ting reticulum cells were present From the data presented t is suggested that the abnormal chromosome complement belonged to the neoplastic reticulum cell

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Primary Malignant Melanoma of the Vagina
A Case Report

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Primary malignant melanomas of the vagina are extremely rare. Ehrmann *et al.* reviewed the literature in 1962 and found 34 cases reported. They added one of their own, which was an amelanotic malignant melanoma. Of the seven apparently diagnosed cytologically,[1] only one contained pigmented cells. Koss[2] reported a case of vulvar melanocarcinoma where pigmented cancer cells were found in the vaginal smear. An *in situ* melanoma was found in the vagina on subsequent resection. The case being reported was not diagnosed by smear, but the cells shed were pigmented and correlated beautifully with those in the histologic sections.

CASE REPORT

The patient was a 58 year old white female, para 2-0-0-2, whose presenting complaints were vaginal discharge and some blood staining. Pelvic examination was not diagnostic and a cervical-vaginal smear taken at that time showed only mild squamous atypia. Because of the symptoms, a D & C was contemplated. The patient was admitted to the hospital nine days later. During pelvic examination under anesthesia, a black sessile papillomatous lesion, 1 cm in diameter was seen on the anterior vaginal wall. The mucosa around the lesion, over an area 1.5 × 5 cm, was pigmented black. The papilloma was removed, the cervix biopsied and curettage of the endometrial cavity was unsuccessfully attempted. Pathologic diagnosis of the pigmented lesion was malignant melanoma, spindle cell type, with ulceration and deep invasion (Fig. 1). No other pigmented lesions were found on the skin.

A cervical-vaginal smear taken 68 days postoperatively showed pigmented malignant cells. Pelvic examination at that time revealed that the mucosal pigmentation was still present. There was slight thickening on the right side of the vaginal wall. Six months after the positive biopsy an anterior pelvic exenteration was performed. An ileal loop with ileo-ureterostomies was also done. Obturator lymph nodes, bilaterally, contained metastases. The patient's post-operative course was uneventful but she died two and one-half months post surgery.

CYTOLOGIC FINDINGS

At first glance, the malignant cells resembled reactive endocervical cells, but on further scrutiny they showed malignant criteria: nuclear membranes were sharp and distinct, nucleoli were blue or bluish-red, enlarged and irregular; chromatin was clumped; there was some chromatin clearing (Fig. 2). The nuclear-cytoplasmic ratio was increased in many but not all of the cells. Many cells contained brown pigment, either in many fine or sparse coarse granules (Figs. 3 and 4). Cytoplasm appeared wispy and was greenish-blue. The cell shapes were irregular, some appearing like distorted spindles, resembling those in the *histological section (Fig. 5). The appearance of the* shed melanoma cells corresponds to descriptions of similar cells shed from other areas.[4, 5]

DISCUSSION

Cytology apparently is not always helpful in the early diagnosis of malignant melanomas. Graham[3] states that malignant melanoma in the vagina

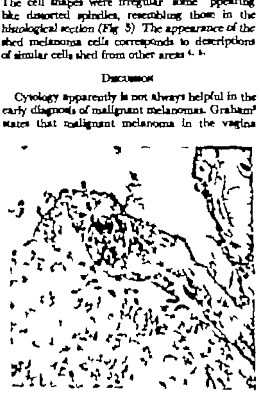


FIG. 1. Histologic section of the tumor showing ulceration. Melanoma cells are invading the epithelium as well as the subcutaneous tissue. H & E stain (X80).

Fig. 2. Cells in the cervical vaginal smear showing malignant criteria. An occasional melanin granule can be seen. Papanicolaou stain (×550)

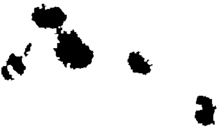
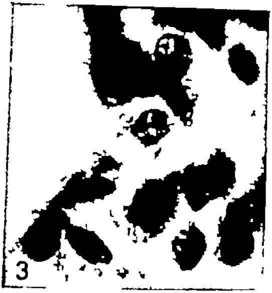


Fig. 4. A cell in the vaginal smear containing one coarse melanin granule. Papanicolaou stain (×320)

does not seem to desquamate cells. However, Loughlin[4] *et al* studying malignant melanomas of the skin found that diagnostic tumor cells could be obtained from direct imprints taken from ulcerated areas of the tumors. They emphasized that poor cohesion of the melanoma cells was the reason for obtaining such a large number of malignant cells. Medek *et al* also reported a case of an oral melanoma diagnosed by cytologic smear but the tumor had been traumatized by previous needle aspiration. Despite the fact that the tumor in this case was ulcerated, no tumor cells were found in the cervical-vaginal smear prior to biopsy.



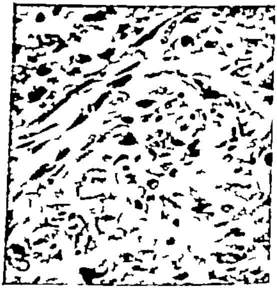


Fig. 3. Cells in the vaginal smear showing fine melanin granules and an occasional coarse one. Papanicolaou stain (Oil immersion ×800)

Fig. 5. Histologic section of the tumor showing melanoma cells having fine melanin granules in the cytoplasm. Note spindle shapes of numerous cells and malignant nuclei resembling those seen in figures 2 and 3. H & E stain (×320)

SUMMARY

The cytologic and histologic findings of a primary malignant melanoma of the vagina are reported. The tumor was not diagnosed by cervical-vaginal smear. The difficulty in diagnosing malignant melanomas of the vagina is discussed briefly.

ACKNOWLEDGMENT

The author wishes to thank Dr Theodore Kardash for allowing him to report the case, and Dr W Bradley King, J pathologist t Maryland General Hospital, for supplying him with histologic section of the tumor

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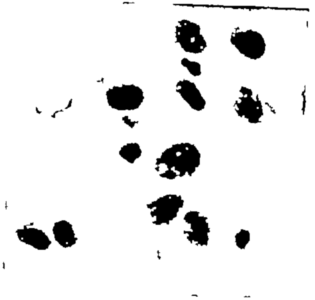

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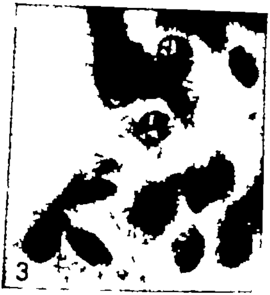

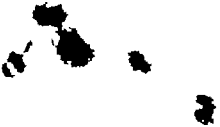

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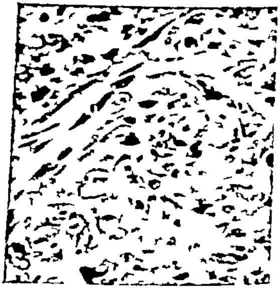

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SUMMARY

The cytologic and histologic findings of a primary malignant melanoma of the vagina are reported. The tumor was not diagnosed by cervical-vaginal smear. The difficulty in diagnosing malignant melanomas of the vagina is discussed briefly.

and source of bleeding (hemorrhagic colpitis, occult bleeding from the endocervical canal.

4. Searching for tumor cells in the vaginal or cervical secretions (search for incipient cancer).
5. Determining the phase of the menstrual cycle by testing for the crystallization phenomenon of cervical mucus (Fern-Leaf test).
6. Studying the mobility of sperm in cervical secretions (Sims-Huhner test).

In addition, the method may be used for:

7. Evaluating corpuscular constituents in urine and urinary sediment.
8. Studying the fluid obtained from ascites or from ovarian cysts.

This rapid method serves ideally as a guide and is especially worthwhile for repeated checkups. The advantages are that the results of the cytologic study are available while the patient is present, the results supplement the clinical and colposcopic studies and may be used directly for deciding treatment. Since prolonged and intensive examination during office-hours is impossible, we prefer to use stained smears for searching for carcinoma cells (the laboratory method). If the Papanicolaou test proves negative, a repeat test is not required for one year. During the interval, check-up examinations may be carried out with the rapid method. Consequently, the time-consuming studies of the cytologic laboratory are circumvented. The use of both methods together represents an ideal in modern medicine. The definitive, rapid, and easily performed method of examination can be applied directly to the patient to provide results which may be used for instituting prompt therapy and results which may be checked by a specialist (cytology laboratory), if such is deemed necessary.

Advantages of vital cytology in the gynecological practice (Condenser U7). R. Gimes. Univ Hosp. I Gyn Dept of Semmelweis University Budapest, Hungary

Using vital cytology, the gynecologist can make quick hormonal diagnosis during the patient's visit. From the cytologic specimen the physician is able to evaluate both the ovarian function and the vaginal microbiology. Clinical diagnosis supported by cytologic findings enables the physician to apply promptly the appropriate hormonal treatment. The more properties of the cells are observed, the more reliable diagnosis can be established. The best result could obviously be achieved by examining the same specimen with different microscopic methods without changing the field of view and with minimum of readjustment. In this paper a new microscopic method is presented: the use of "Condenser U7". It allows the specimen to be examined in succession with an Abbé type condenser under dark ground conditions and also with peripheral illumination, phase contrast, color contrast, complementary contrast, and three-dimensional method. Application of the "Condenser U7" which is suitable for examining fresh unstained and stained specimens alike, is—in our opinion—of great importance in scientific and technical work.

Electron microscopy in diagnostic cytology of serous effusions. P. Pfitzer and F. Huth. Pathologisches Institut der Universitat, Dusseldorf, Germany

For amplifying diagnostic cytology we investigated 29 serous effusions from 26 patients by electron microscopy. The material consisted of 5 inflammatory exudates, 3 systematic diseases (1 lymphoma, 1 reticulosis, 1 plasmocytoma), 3 non-epithelial tumors (1 melanoblastoma, 2 seminoma), 3 bronchial carcinomas, 4 carcinomas of stomach and intestine, 1 carcinoma of portio uteri, and 7 ovarian carcinomas. The electronmicrographs support the diagnosis of malignant criteria of single cells mainly by revealing nuclear polymorphism and changes of cell organelles as well as their atypical localization. The origin of cells can be traced by signs of differentiation, for example, in cases of reticulosis, plasmocytoma, and seminoma. Coherence of cells by desmosomal junctions can be differentiated from accidental clotting and degenerative vacuolization from mucin or phagosomes. Melanotic cytosomes differ di- phagosomes. Melanotic cytosomes differ di- microscopic investigation can be performed within 3 or 4 days and completes the cytologic diagnosis in critical cases.

The visual proof of the penetration of Döderlein bacillus in the exfoliated vaginal cell by the electron microscope. J. Židovsky and Š. Gašpar Research Institute for Care of Mother and Child, Prague, Czechoslovakia.

The *original* method of selective detection of exfoliated vaginal cells in the electron microscope was used, *which* enabled us to examine cells selected in advance by a light microscope. This method allowed us to observe the lytic activity and penetration of Döderlein's bacilli into the exfoliated cellular substrate. This proof is impossible to achieve with the conventional light microscope. Thus the doubts about the cytolytic activity of this microbe were excluded and the findings of Wied and Christiansen fully confirmed.

Electron microscopic study of circulating cancer

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ABSTRACTS FROM THE FIRST SYMPOSIUM ON CYTOLOGY, PRESENTED AT THE FIRST MEETING OF THE EUROPEAN FEDERATION OF CYTOLOGY SOCIETIES

Prague September 1970

Editors J Zidovsky J Jenny

I New and Little Used Methods in Cytology

Phase contrast microscopy H K. Zinser Evangelisches Krankenhaus, Köln/M Germany

Phase contrast microscopy in conjunction with microcinematography plays an important part in experimental cytology for illustration and continuous observation of activities of living objects. The exhibition of phases of cell division registration of growth phenomenon in the tissue culture and the influence of cytostatics on tumor explants are just a few of the various applications.

In applied cytology phase contrast microscopy has not attained the position which it deserves. This is due to the fact that results are preponderantly taken from living or dried material, just immediately after they have been obtained in the practice of a doctor or as used in a clinical laboratory The examining doctor must therefore be a cytologist at the same time when no other cytologist is available Under certain presuppositions this procedure represents an essential enrichment to the cytodiagnostic method First, the procedure has stood the test in proving and differentiating of protozoa, fungi and bacteria It is therefore suitable in gynecology especially for diagnosis of normal and pathogenic vaginal flora and also for the recognition of trichomonad infestation and monilinia attacks. Phase contrast microscopy is also indispensable in the observation of mobile objects. This is clearly seen during the execution of the ascention test (from Sims-Huhner) and the penetration test (from Miller Kurzrok) respectively The method could also be used in judging or assessing the fern-leaf test since crystallization processes can impressively be followed with its aid. For cytologic work on all body fluids, secretions, punction fluids and tumor preparations, this microscopic procedure could in every possible way be applied The main advantage does not lie in the saving of time but in the outlook of the observed objects which in fact undergo no treatment like fixation or coloring

In normal and pathologically changed objects certain structures can be identified which normally in absorption, escape.

The importance of vital cytologic consideration is discussed from several fields of applied cytology

Finally references are made to the advantages of phase optical pictures in the examination of dyed smears.

Cytology at the doctor's office (phase contrast microscopy). P Stoll, Frauenklinik im Klinikum Mannheim der Universität Heidelberg, Germany

The clinician who has a basic knowledge of cytology should be able to evaluate a smear during the patient's visit. Phase contrast microscopy has proved its value for "the rapid diagnosis during office hours With the use of this microscope a quick inspection of a vaginal smear may be made while the patient is still on the examining table The technique of phase contrast microscopy is especially suited for

1 Evaluating ovarian function from the vaginal epithelial cells.
2 Obtaining an impression of the different microorganisms in the vagina. The following organisms may be readily differentiated.
   a) The Döderlein's bacillus.
   b) Trichomonads.
   c) Fungal infections (vaginal mycosis, especially Candida albicans).
   d) Hemophilus vaginalis.
   e) Mixed flora
3. Evaluating polymorphonuclear leukocytes and erythrocytes in vaginal and cervical secretions, when these are taken separately with the aim of localizing the inflammation (vaginitis, endocervicitis, endometritus).

their importance in the diagnosis of pulmonary disease. G. Sesey-Dobray. Spital János, I. Lungen-Abteilung und Bronchocytol. Labor, Budapest, Hungary

Sputum cytology examinations, depending on their number, afford diagnoses in 50 to 80 per cent of cases. They are particularly useful for exfoliating lung cancers, but only bring results in 40 to 50 per cent of peripheral cancers. If exfoliative cytology fails to bring results, various biopsy methods have to be tried in order to obtain material for histologic or cytologic examinations. Histologic material can frequently not be obtained in pulmonary diseases, mainly in the diagnosis of lung tumors, or only at the risk of major intervention. A small amount of material is sufficient and can be obtained for cytologic examinations with a thin needle with less risk.

Of 152 needle biopsy specimens, 100 punctates were assessed (48 transthoracic lung punctures, 31 lymph-node punctures, 12 carina and 9 other punctures). In 80 per cent of the cytologic examinations, the diagnosis was correctly positive or negative. In 57 per cent cytology alone ensured the diagnosis (57 % in tumors).

Depending on the diagnostic problem, all types of puncture cytology can be used successfully even for graver cases as the patient is put to little danger or discomfort. Depending on the diagnostic problem and the facilities, cytologic examinations can be carried out with histological tests or separately. Puncture cytology leads to a diagnosis in some cases even where exfoliative cytology fails to bring results and a histologic test cannot be performed. A cytologic diagnosis can decisively affect the selection of the therapeutic method to be adopted.

Coordinated comparative cytologic, histologic and cytogenetic studies of the cervix uteri. (Results and conclusions). F. Dehnhard and H. Breinl. Sektion Zytol. u. Histol. der Universitäts-Frauenklinik, Ulm, Germany

Both on the basis of the few results currently available from other authors, and also with reference to our own findings, new possibilities for the application of chromosome examination in prognostic diagnosis of premalignant lesions of the cervix uteri are discussed.

The incidence of precancerous lesions of the uterine cervix in relation to different type of vaginal inflammation. A. Králová. Postgraduate Medical Institute, Prague, Czechoslovakia

Two hundred seventy-five women suffering from trichomoniasis, bacterial or mycotic colpitis were examined and followed for five years after antiphlogistic treatment by means of cytology and colposcopy. The prebioptic findings before and after the corresponding treatment were compared. The persisting suspicious or positive findings were proved by histology and the incidence of carcinoma in situ and other precancerous lesions in relation to the different types of treated vaginal inflammation was stated.

Diagnosis of symptomless endocervical microcarcinoma. J. Gorca, L. Brella and F. Hargitai. Laboratory of Cytology, the Municipal Hospital of Pécs, Pécs, Hungary

The fate of patients with preclinical cancer of the uterine cervix diagnosed at Pécs from 1950 to 1955 is presented. Of the 101 patients in stages "O" and Ia 96 (95%) were alive, and still followed after 5 years, and 91 (90%) after 10 years.

In the Municipal Hospital of Pécs 50,000 colpocytologic examinations have been performed over the past 10 years in 25,000 women. In 34 of them stage "O" of cancer of the uterine cervix was diagnosed, in 21 stage Ia and in 5 stage Ib. In 42 cases colposcopy and colpocytology together or separately indicated malignancy unambiguously. In 8 cases the diagnosis could only be inferred, solely from endocervical smears. Two cases will be reported in detail. It should be emphasized that after the use of a grooved probe in gynecologic cancer screening above the age of 35 secretion should be taken also from the endocervix in every case. This method yielded the cells indicative of malignancy (P V), even though a simple colpocytologic examination has failed to demonstrate them.

The possibility of cytologic prediction of carcinoma in situ of the cervix. S. Audy, J. Ivić and P. Begović. Department of Gynecology and Obstetrics, Medical Faculty, University of Zagreb, Yugoslavia.

The authors discuss the possibility of predicting histologic diagnosis of cervical carcinoma in situ on the basis of cytologic analyses in 200 cases.

Qualitative cytomorphologic criteria have been used in establishing cytologic diagnosis.

Because, the cytologic prediction of histopathologic diagnosis might have a direct influence on further diagnostic and therapeutic procedures, its precise value has to be evaluated.

The authors are of the opinion that this goal may be achieved if collaboration between the cytologist, the clinician and the histopathologist is in existence and made closer and closer.

The epidemiological aspects of cytologic screening for cervical carcinoma in Ljubljana. J. Koralič and S. Borta. University Teaching Hospital for Obstetrics and Gynecology, Ljubljana, Yugoslavia.

This report deals with the treatment of 98,144 *cells.* L. Döbrövy and J. Tóth. Oncopathological Research Institute Budapest Hungary

Light microscopic study of circulating cancer cells seems to be suitable for verifying the presence and number of the tumor cells recovered from the peripheral blood but their integrity or disintegration could be reliably identified only by ultrastructural study. Our observation has shown that during the process of hematogenous dissemination a considerable portion of circulating tumor cells are intact in their fine structure. These are viable and able to proliferate, the other portion of isolated tumor cells are seriously disintegrated. This is probably why all malignant cells circulating in the blood stream fail to rise to blood borne metastasis.

Comparing light microscopic and electron-microscopic observations on cells of an undifferentiated small cell carcinoma. S. Zimmer. Zytodiagnostisches Zentrallaboratorium Grimma Germany

An undifferentiated bronchogenic carcinoma of small cell type was detected by sputum cytology in the absence of roentgenologic findings.

Microphotographs originating from the sputum cytology of this case, from dab smears taken from material of lung resection and pictures of histologic sections were taken.

Two years after pneumonectomy a lymph node above of the clavicle was punctured. The smears revealed metastasis of the bronchogenic small cells carcinoma.

The material taken from lung resection was also examined by electron microscopy.

Some additional criteria of small cell carcinoma as seen by electron microscopy are: dark cells with hyperchromatic nuclei and narrow cytoplasm, few light cells with transparent nuclei and wider cytoplasm, a few mucous cells with Golgi apparatus, condensed chromatin clumps, irregularly distributed chromatin condensation along the nuclear membrane, nucleoli with undeveloped nucleolonema, irregular nuclear pores, lobulations and cytoplasmic invaginations, a few mitochondria only, may be dense or empty cisternae with Serotonin granules, endoplasmic reticulum is scanty, no desmosomes, no tonofibrilis, and interdigitations of some microvilli only.

In spite of electron-microscopic research no mark of Cain of cancer cells could be found.

The new method for study the hormonal changes in rat urinary bladder. J. Červenka, A. Kotásek and M. Brešťák. Department of Gynecology and Obstetrics of Pediatrics Faculty of Charles University Prague Czechoslovakia.

The authors used adult female rats, strain Wistar. The rats were fed the standard food, and kept in standard conditions (dark-light). After the necessary adaptation vaginal smears were taken for at least two cycles and only those having normal estrous cycles were elected. Under ether narcosis, laparotomy and resection of part of the abdominal wall was done, the urinary bladder was fixed to the periphery of the laparotomy and resection of one half of the bladder was performed. After two weeks the borders of the bladder had healed. Smears were taken simultaneously from vagina and from the surface of the base of the urinary bladder after one week. The smears were taken with a swab, fixed and stained with the Papanicolaou stain. This operative method was performed in twenty rats. Smears taken from vagina were in agreement with those taken from the urinary bladder. During the estrus, the authors observed a red area in the center of the marsupialised bladder. After this operation no complication was observed. Smears from the bladder were taken for three months.

II. General Aspects on Carcinoma in situ of the Uterine Cervix and of the Endometrium

The advantages of a computerized program of population screening for cervical cancer. E. von Haam. Department of Pathology College of Medicine Ohio State University Columbus, U.S.A.

This represents an analysis of the computerized data of 15,000 Papanicolaou smears from the female genital tract of women predominantly in the child-bearing age. It contains a computerized analysis of the most important data in the history and physical examination of the patient, including the menstrual status, the number of pregnancies, and any previous significant surgical or therapeutic procedures including the use of contraceptives. The prevalence rate of abnormal Papanicolaou smears was higher in women with dysmenorrhea, leukorrhea, menorrhagia, metrorrhagia, or postcoital bleeding. An analysis of the methods of contraception used showed no significant differences between the women who used the pill and women whose methods of contraception were unknown. There was, however, a significant increase of abnormal smears in women using the intrauterine device. There was also a higher prevalence rate of cervical erosion, cervical polyps, and cystic cervicitis in women with abnormal Papanicolaou smears. All cases of carcinoma in situ and invasive carcinoma had either a positive or suspicious cytologic diagnosis.

Methods of punction cytology examinations and in whom thyroid cancer was cytologically proved in the lymph node punctate there was no changed palpatory finding in the thyroid and it was only the lymph node punctate that revealed the existence of thyroid cancer

The authors consider the cytodiagnostic aspiration biopsy of any node of the neck, combined with the knowledge of the cytologic picture of these nodes and of the thyroid, provides a very helpful diagnostic method, because it reveals the nature of pathologic processes in a simple, quick, and reliable way

The diagnostic value of the gastrocytogramm in different gastric diseases. I. Miczbán. Krankenhaus-Poliklinik János, Zentrallaboratorium, Budapest, Hungary

Cytologic results obtained by lavage technique were collated with clinical diagnosis in 885 patients. The diagnostic accuracy of the cytologic investigations was, in the malignant cases, 75.9 per cent and in non malignant cases, 92.4 per cent.

Following the percentual evaluation of the results, the cytologic diagnoses were compared with the results simultaneously obtained by X-ray and gastroscopic examinations.

It has been proved mathematically that the diagnostic chances became significantly better by the combined investigation because of the successful addition and completion each of the three methods contributed.

It has been emphasized also, that the gastrocytogramm is an extremely sensitive indicator of lesions of the gastric mucosa. Thus, the importance of the gastrocytologic investigation is greatly increased beyond its oncocytodiagnostic value. It calls attention to alterations, which, though not malignant in themselves, may be the precursors of malignant processes. Moreover it is suitable for following up the therapeutic effects. It also represents the alterations of the gastric mucosa in diabetics.

The importance of team-work and that of the close co-operation with the clinician is stressed.

It is pointed out, that gastrocytology cannot be used as independent population screening method, but it is a very useful procedure for selected patients with gastric symptoms, adapted conjointly with radiology and gastroscopy

Cytologic diagnosis of adnexal tumors by fine needle biopsy J. Ivič and S. Audy Department of Gynecology and Obstetrics, Medical Faculty University of Zagreb, Yugoslavia.

This report deals with cytologic examinations in 230 cases of adnexal tumors where fine needle biopsy was performed.

In the cases of cystic tumors two different techniques of preparing classical and Sayk modified method of natural sedimentation, and staining the smears (Pappenheim and Papanicolaou) were applied.

According to the cytomorphologic picture, differential diagnosis of various pathologic conditions was established.

The correlation with the histopathologic diagnosis confirmed that the cytodiagnosis of adnexal tumors is an efficient and accurate method for differential diagnosis.

Today the risk of fine needle biopsy resulting in the propagation of an inflammatory process or tumor spread is less negligible.

Aspiration biopsy of thyroid goiter V. Šmejkal and E. Šmejkalová. Research Institute of Endocrinology Prague, Czechoslovakia.

The authors have analysed the results of 1,000 aspiration biopsies of thyroid goiters and described the cytologic patterns of the most common pathologic processes important for clinical diagnosis. The most useful application of this simple and harmless method is in the examination of scintigrafic cold nodes. One can differentiate between benign tumors, chronic inflammatory processes, and cysts. In haemorrhagic cysts (more than 10% in the presented series) the aspiration is a therapeutic measure as well. In the authors material there are 40 malignant tumors, out of which there are 30 carcinomas of the thyroid, 3 metastatic tumors and 7 malignant lymphomas. All cases of cancer in this series differed cytologically so conspicuously from the standard pattern that their malignant character could be recognized. Retrospectively it is possible to discern to a certain extent between the differentiated and anaplastic carcinomas. On the other hand there were a number of false positive diagnoses. Most of these cases were histologically parenchymal adenomas (microfollicular, trabecular, papillary), cold in scintigramm. These histologically benign tumors are not always predictable in their clinical behavior and react well to surgical intervention. Besides the basal Pappenheim method parallel smears were examined, dyed with toluidine blue for the demonstration of nucleoli in thyroid cells. The advantage of the latter stain was demonstration of their morphologic properties, when evaluating their functional as well as growing activity

Diagnostic possibilities of puncture cytology in reference to parametrial findings after radiologic therapy of carcinomas of female genitals. M. Rold, W. Koch, K. Jantzen and R. Labensky U niversitäts-Frauenklinik, Göttingen, Germany

An account is given of 140 punctures with puncturing needle ("Stockholmer Gerät" or "Silbermann-Nadel") in cases of carcinomata of first and 52 193 repeated cytologic examinations in women living within the Ljubljana region in the period 1960 to 1966. The authors deal with the problem of prevalence and incidence of the severe dyplasias and of the intraepithelial carcinoma of the cervix in relation to the age of the women. With purpose of establishing the eventual influence of cytologic detection on the dynamics of cervical carcinoma in the female population the authors also present the incidence of invasive carcinoma of the cervix in the womens population of Ljubljana Beside this the alterations in the structure of stages and mortality of the cervical carcinoma in the period from 1955 to 1967 were also studied.

III Cytology of Effusions and Aspirates. Its Problems and Value in the Clinical Diagnosis

Three-dimensional tumor cell diagnosis (cytology of effusions). B. Choné Universitäts-Strahlenklinik Heidelberg, Germany

Based on cytomorphologic findings and follow up studies of 1,220 malignoma having developed pleural or peritoneal metastases, general directions of diagnostic and therapeutic value were realized. The collection was represented by different primary tumors including cell analysis related to morphologic (panoptic staining), autoradiographic (labeling with ^{3}H thymidine) and cytochemical (Acridine orange) perspective. The H labeling index fluctuates over a great range and is dependent on certain features of the cell structure.

There is evidence to the following conclusions.

1) Type site and histologic classification of primary tumor has no visible influence on the proliferation activity of metastases.
2) The knowledge of differences in proliferation kinetics obtained by in vitro or in vivo inculation of effusions with tritiated thymidine is the best guarantee for effective therapy
3) In combination with cytochemical methods—especially of the vital staining group—a further parameter is provided as a functional indicator of the tumor cell population This results in a three dimensional form of consideration.

Cytologic and histologic comparative study in mesothelial serous effusions C. Marsan and J Roujeau. Service Central d Anatomie et Cytologie Pathologiques, Faculte de Medicine Lariboisiere Paris, France

The purpose of the present study was to determine whether a cytologic and histologic comparison by means of serous effusion smears and pleural or peritoneal tissue sections (biopsy or autopsy) might improve cytologic diagnosis. Seventy two cytologic-histologic cases were compared

The smears were stained according to the method of Papanicolaou careful collection was of importance for accurate diagnosis. The sections were prepared in the usual way

The cytologic patterns are successively diagnosed in cases of inflammatory fluids in which a mesothelial cellular type and a mixed cellular type are distinguished, containing more or less atypical mesothelial cells, the identification of which may be difficult, and malignant fluids in which monomorphal and polymorphal cellular patterns are also distinguished

The histologic study follows the same plan. It is contended that inflammatory changes can be grouped into general rules of inflammation and that inflammatory patterns may also result in diagnostic difficulties.

Both cytologic and histologic series are compared showing that the mesothelial cellular type is always connected with an inflammatory disease whereas the polymorphal type while more often connected with such a disease, may also be dependant on a tumoral growth. This justifies some of the pitfalls in cellular diagnosis, at least that effective neoplastic cells are discovered in 77 per cent of pleural fluid but only in 60 per cent of ascitic fluids.

The authors conclude that histologic and cytologic comparison is of value because of persisting difficulty in identification of mesothelial cells and cytologic patterns. They suggest that, by contributing to a better understanding of cellular changes, it probably will reduce the percentage of suspicious and false positive cases and will serve as a guide to more effective diagnosis.

Detection of thyroid carcinoma by the cytodiagnostic puncture of lymph nodes of the neck M Dominis, I Črepinko and Z Skrabalo. Department of Medicine "O Novosel" Medical School, University of Zagreb Zagreb Yugoslavia.

Although no specific laboratory analysis allows an early diagnosis of thyroid tumors, scintigraphy and cytodiagnosis do help a great deal in the detection of this kind of tumor The authors set forth the results of 1 532 punctures of the lymph nodes of the neck made from 1962 to 1969. Carcinomatous metastases were cytologically evidenced in 86 patients. Out of them, 11 were cytologically proved to have metastases produced by thyroid cancer In the same period, by the cytologic analysis of 5 893 thyroid punctates, thyroid cancer was evidenced in 55 patients. All cytologic findings were later varified histologically It should be pointed out that in 6 patients in whom thyroid cancer was cytologically proved in the lymph node punctate, there was no changed palpatory finding in the thyroid and it was only the lymph node punctate that revealed the existence of thyroid cancer

The authors consider the cytodiagnostic aspiration biopsy of any node of the neck, combined with the knowledge of the cytologic picture of these nodes and of the thyroid, provides very helpful diagnostic method, because it reveals the nature of pathologic processes in simple quick, and reliable way

The diagnostic value of the gastrocytogramm in different gastric diseases. L. Mierbán- Krankenhaus-Poliklinik János, Zentrallaboratorium, Budapest, Hungary

Cytologic results obtained by lavage technique were collated with clinical diagnosis in 883 patients. The diagnostic accuracy of the cytologic investigations was, in the malignant cases, 75.9 per cent and in non malignant cases, 92.4 per cent.

Following the percentual evaluation of the results, the cytologic diagnoses were compared with the results simultaneously obtained by X-ray and gastroscopic examinations.

It has been proved mathematically that the diagnostic chances became significantly better by the combined investigation because of the successful addition and completion each of the three methods contributed.

It has been emphasized also, that the gastrocytogramm is an extremely sensitive indicator of lesions of the gastric mucosa. Thus, the importance of the gastrocytologic investigation is greatly increased beyond its oncocytodiagnostic value. It calls attention to alterations, which, though not malignant in themselves, may be the precursors of malignant processes. Moreover it is suitable for following up the therapeutic effects It also represents the alterations of the gastric mucosa in diabetics.

The importance of team-work and that of the close co-operation with the clinician is stressed.

It is pointed out, that gastrocytology cannot be used independent population screening method, but it is a very useful procedure for selected patients with gastric symptoms, adapted conjointly with radiology and gastroscopy

Cytologic diagnosis of adnexal tumors by fine needle biopsy J I R and S. Audy Department of Gynecology and Obstetrics, Medical Faculty University of Zagreb, Yugoslavia.

This report deals with cytologic examinations in 230 cases of adnexal tumors where fine needle biopsy was performed.

In the cases of cystic tumors two different techniques of preparing classical and Sayk modified method of natural sedimentation, and staining the smears (Pappenheim and Papanicolaou) were applied.

According to the cytomorphologic picture, differential diagnosis in various pathologic conditions was established.

The correlation with the histopathologic diagnosis confirmed that the cytodiagnosis of adnexal tumors is an efficient and accurate method for differential diagnosis.

Today the risk of fine needle biopsy resulting in the propagation of an inflammatory process or tumor spread is less negligible

Aspiration biopsy of thyroid goiter V Šmejkal and E. Šmejkalová. Research Institute of Endocrinology Prague, Czechoslovakia.

The authors have analyzed the results of 1,000 aspiration biopsies of thyroid goiters and described the cytologic patterns of the most common pathologic processes important for clinical diagnosis. The most useful application of this simple and harmless method is in the examination of scintigrafic cold nodes. One can differentiate between benign tumors, chronic inflammatory processes, and cysts. In haemorrhagic cysts (more than 10% in the presented series) the aspiration is therapeutic measure as well. In the authors material there are 40 malignant tumors, out of which there are 30 carcinomas of the thyroid 3 metastatic tumors and 7 malignant lymphomas. All cases of cancer in this series differed cytologically so conspicuously from the standard pattern that their malignant character could be recognized. Retrospectively it is possible to discern to certain extent between the differentiated and anaplastic carcinomas. On the other hand there were a number of false positive diagnoses. Most of these cases were histologically parenchymal adenomas (microfollicular, trabecular, papillary), cold in scintigramm. These histologically benign tumors are not always predictable in their clinical behavior and react well to surgical intervention. Besides the basal Pappenheim method parallel smears were examined, dyed with toluidine blue for the demonstration of nucleoli in thyroid cells. The advantage of the latter stain was demonstration of their morphologic properties, when evaluating their functional as well as growing activity

Diagnostic possibilities of puncture cytology in reference to parametrial findings after radiologic therapy of carcinomata of female genitals. M Röhl, W Koch, K. Janssen and R. Lebensky Universitäts-Frauenklinik, Göttingen, Germany

An account is given of 140 punctures with a puncturing needle ("Stockholmer Gerät" or "Silbermann-Nadel") in cases of carcinomata of the female genitals after radiologic treatment. *Parametrial findings* have been punctured by vaginal or rectal routes to clarify the diagnosis of recidive. Results of cytologic examinations are reported. Difficulties of this method and complications are pointed out. Therapeutic consequences and results of development are reported.

Cyto-histologic correlation between punctates and testis biopsy material. I. Črepinko, M. Dominis, J. Posinovec and Ž. Skrabalo. Department of Medicine "O. Novosel" Medical School, University of Zagreb, Zagreb, Yugoslavia

In testicular tissues obtained by cytodiagnostic puncture or biopsy and stained with May Grünwald-Giemsa it is possible to cytologically analyze the morphology of testicular cell elements. In addition to histologic findings, cytologic analysis of the testis has in the last 8 years been used for the study of male sterility. This *paper presents the cytologic pattern of* 152 testes of persons with functional gonadal disorders and correlation between cytologic and histologic findings. In 40 persons, cytologic and histologic analyses were performed in both testes as it is known that morphologic changes in one testis often differ from those in the other.

The interpretation of the cytologic picture was made 1) by observing both morphologic appearance and amount of spermatogenetic cells, 2) by determining the percentage of sperms by counting 100–200 spermatogenetic cells, and 3) by observing the morphologic appearance and amount of other cells (*i.e. Sertoli's and Leydig's* cells and the amount of fibrocytes).

According to these criteria the cytologic findings were divided into 4 groups (A, B, C, D) and *histologic findings in 5 groups (I–V)*. Comparing cytologic and histologic material, they were often found to be in good agreement and even supplementing each other in some cases. Cytologic analysis for carbohydrates, RNA, DNA and acid phosphatases has shown that the most pronounced alternations occur in Sertoli cells, the morphologic picture of which is considerably changed, corresponding to the cytologic C and D and histologic III–V groups.

On the basis of their experience the authors *consider* the cytologic analysis of the testis, with regard to its simple application very suitable *especially in cases* when morphologic changes in the testicular tissue of persons with functional gonadal *disorders require* frequent examination during the therapy.

Intraocular diagnostic cytology—primary cytodiagnosis of retinoblastoma. N. Sagiroglu and T. Örgönül. Department of Cytopathology and Cancer Research, Hacettepe University, Faculty of Medicine, Ankara, Turkey

To evaluate the cytodiagnosis of intraocular fluid in clinics of ophthalmology a series of smears were prepared. Two cases among children were diagnostic problems in the Department of Ophthalmology and we were asked whether a cytologic evaluation would be possible *or not.* Ophthalmologists were advised and encouraged by us to obtain intra-ocular fluid by needle aspirations then, bring them immediately in the syringes to our Cytopathology and Cancer Research Department. This was accomplished under general anesthesia and sufficient fluid was obtained in each case.

In the laboratory several smear slides were prepared. The specimens were fixed by spraying with dry fixative then stained according to Papanicolaou, toluidin blue and P.A.S. staining *techniques. In addition to these* fresh material was also studied under dark field and by coloring with the TMK 101 Turk, a polychrome supravital staining procedure. Cytodiagnosis was made in the first case, as "benign fibrocytic cells and histiocytes filled by lipid inclusions, suggestive of a benign tumor or granulomatous disease" and in the second case "Malignant cells, conclusive of retinoblastoma".

Fifteen days after the cytodiagnosis, biopsy of the first and enucleation specimen in the second case had confirmed the cytodiagnosis.

Upon these successful results, a series of experimental cytology specimens were prepared from the intraocular fluid obtained from patients *during corneal* and lenticular cataract surgery. These specimens have shown the normal glandular like *cells exfoliated* physiologically into the fluid of camera anterior.

From these observations *it was concluded* that intraocular fluid aspirations and cytologic studies will be a useful diagnostic procedure in the clinics of Ophthalmology.

Cytology of liquor amnii. O. Nyklíček and I. Hradil. Department of Gynecology and Obstetrics, Náchod, Department of Histology and Embryology, Faculty of Medicine, Hradec Králové, Czechoslovakia.

The authors describe various kinds of epithelial cells in amniotic fluid, as seen during the stages pregnancy. The smears were stained according to the Harris-Shorr method and Nile blue staining. This study was undertaken in an attempt to identify the origin of cells in liquor amnii. For this reason the cells in smears from amniotic fluid were compared with those in smears undertaken from the surface of amnion, umbilical cord and the fetal skin. These smears were then compared with histologic patterns of appertaining epithelial organs.

The cytology of amniotic fluid by Nile-blue staining. M. Khrrich. Research Institute for the Care of Mother and Child, Prague, Czechoslovakia.

An original cytologic method investigated by the author is presented. The principles of the method are: vaginal smears taken on two slides are stained without drying or fixation by means of the 1% water solution of Nile blue sulphate. After 3 to 5 minutes the preparations are covered slipped and examined under the microscope. Orange stained elements are fetal skin cells or drops of fetal skin fat. The other non fetal cellular elements are blue stained. The accuracy of this method for the diagnosis of ruptured fetal membranes is higher than 98 per cent after the 32nd week of pregnancy. The method is, in comparison with other cytologic diagnostic methods, simpler and quicker and therefore very useful for obstetric practice.

In the second part of the paper experiences with the cytologic evaluation of the maturity of the fetus from the amniotic fluid are presented. The technics of Brosens and Gordon is as follows: 500 cells of amniotic fluid smear (made of 1 drop of amniotic fluid and 1 drop of Nile-blue sulphate) are counted under microscope. More than 10 per cent of orange stained cells of total number proves the maturity of the fetus. This positive result is very reliable. Less than 10 per cent of orange stained elements is not proof of the immaturity of the fetus. According to the author's experience the method could not be useful for the diagnosis of fetal postmaturity.

Cytologic estimation of the sensitivity of the tumor to chemotherapy in cases of advanced ovarian tumors. O. Drobík. Oncological Laboratory of the Medical School of Charles University, Prague, Czechoslovakia.

Cytologic changes in ascitic and pleural fluids in advanced cases of gynecologic cancer during chemotherapy are described. All smears were positive before the initiation of treatment.

The favorable effect of cytostatics on malignant growth is manifested by an increased number of atypical elements. The nuclei are larger, poorly stained, sometimes strikingly hypochromatic and a fine vacuolization occurs in the cytoplasm. These signs of degeneration culminate in the form of karyolysis. In cases where the cytostatic has lost its inhibitive effect the number of degenerative changes decreased significantly. At this time it was necessary to apply a new cytostatic drug.

The cytostatic agent exhibiting the greatest inhibition of ^{125}I Iododeoxyuridine incorporation in vitro was recommended for the treatment. The results of 70 cases demonstrate that selective chemotherapy of ovarian cancer with ascites is possible. The newly applied cytostatic with another effective mechanism again caused fresh signs of cellular degeneration.

Cytologic diagnosis of tuberculous mastitis. Z. Radojkov, I. Berger and D. Ristic. Institute of Pathologic Physiology—Medical Faculty Institute of Pathologic Anatomy—Clinical Hospital, Central Clinical Laboratory—Clinical Hospital, Novi Sad, Yugoslavia.

Using cytologic examination of tumefaction aspirates and fluids of male and female mammary glands (the latter taken during the lactation period), the authors established the diagnosis of tuberculous mastitis. Of 1,600 examinations of different proliferative and inflammatory processes of mammary glands, the suspicion of tuberculous mastitis had been established in 8 patients (0.50%). In 4 cases (0.25%) the cytologic diagnosis had been confirmed by the bacteriologic and pathohistologic examinations.

By the cytologic examination of smears of aspirates and fluid of mammary glands it is possible to find different cytologic patterns. Cytologic findings in this disease depend on the degree of the tuberculous process. Cytologic pattern typical for granulomatous inflammatory process with numerous lymphocytes, epithelioid and giant Langhans cells, plasmocytes etc. or the cytologic signs of cellular necrosis and copious cellular detritus in the stadium of the beginning caseous necrosis were found.

In the paper the cytologic differencial diagnostic is presented for nonspecific chronic mastitis of different etiology, plasmacellula mastitis and secondary inflammatory processes in the course of blastomatous and others proliferative processes.

The paper is illustrated with original microphotographies of smears of aspirates and fluid of mammary gland.

Studies on the problem of cytologic diagnosis of the type of pulmonary carcinoma by comparative cytologic and histpathologic examination. W. Ebert and Ch. Engel. Pathologisches Institut der Friedrich Schiller Universitat, Jena, Germany.

In many cases of malignant lung tumors it is impossible to obtain material for histopathologic examination because of technical reasons or inaccessible localization of the suspected tumor. Therefore cytologic examination of the sputum, bronchial secretion or lung punctures are often the only possibility for morphologic tumor diagnosis. If tumor cells or groups of tumor cells are found, diagnosis of the type will be of interest because of its consequences for therapy and prognosis. The possibilities of cytologic classification of pulmonary carcinoma by confrontation of histopathologic and cytologic findings was attempted. It may be pointed out that it is usually possible to classify the cases of keratinizing and non-keratinizing squamous cell carcinoma with very high probability of correctness. Contrarily the diagnosis of undifferentiated carcinoma occuring in the pattern of polymorphocellular or so-called parvicellular respiratory oat-cell carcinoma is considered difficult. In such cases diagnosis has only a slight degree of probability. It may be possible to succeed in distinguishing polymorphocellular carcinoma from parvicellular carcinoma but otherwise it is scarcely possible to distinguish parvicellular carcinoma from oat-cell carcinoma. In adenocarcinoma occurence of adenoid layered cell groups and occasionally secretory appearances in cells are characteristic features. Often it is difficult *to distinguish cells originating from pulmonary* adenomatosis or in alveolar cell carcinoma from adenocarcinoma cells. Occurrence of alveolar cell carcinoma based solely on cytologic findings *ought to be suspected only if the tumor cells* reveal the exact characteristics. Also in well preserved specimens and completely matured tumor cells cytologic classification of pulmonary *carcinoma must be interpreted with necessary* caution and criticism as sometimes different structures are associated in malignant lung tumors. Furthermore differential diagnosis me*tastases in connection with the bronchial sys*tem must be taken into consideration.

Cytology of transrectal prostatic aspirations. H. Lederer and H. Sunderland. Doncaster Royal Infirmary, Doncaster, England, U.K.

Franzen's method of cytologic examination of prostatic aspirates is not widely practiced in this country although fully established in Sweden and reported to be painless, free of complications and practicable as an outpatient procedure.

The present study was undertaken in order to evaluate the method and its application as a routine procedure in a District Hospital.

Aspiration smears were examined from 34 prostates obtained by prostatectomy for urinary obstruction and from 89 unselected consecutive autopsies in the age group over 50 years. These were compared with paraffin sections of the organ. In addition 87 smears were obtained from 80 patients. Histologic confirmation was possible in 23 cases of this series. No false positive or false negative results have been obtained. However a fairly high proportion of doubtful (8%) and inadequate (19%) smears are recorded. It is expected *that this proportion* will fall with more experience.

The study indicates that transrectal fine needle prostatic aspiration biopsy is a safe method and a valuable diagnostic help.

Transthoracic cytodiagnostic aspiration puncture. H. Harambasic, E. Hauptmann, V. Pelut and M. Roglić. Department of Medicine "O. Novosel" Medical School, University of Zagreb and the Institute for Tuberculosis and Lung Diseases, Zagreb, Yugoslavia

The authors have in the last two decades performed about 900 transthoracic cytodiagnostic aspiration punctures of various pathologic changes in the lungs and mediastinum. The results obtained with particular reference to the percentage of the punctured material suitable for further analysis, the percentage of correct and false-positive diagnoses, and complications, are presented as well as the authors views on indications and contraindications for transthoracic cytodiagnostic aspiration puncture.

I. New and Less Used Methods in Cytology

Cytologic diagnosis by fluorescence. C. Conti and P. Haour. *Centre Anti-Cancéreux de* Lyon, Lyon, France.

Many years ago, we began to use fluorescence as a complement to the Papanicolaou technic, *in order to improve the diagnostic accuracy of* difficult or borderline cases.

We were interested in the advantages of this technic and the first results were so satisfying *that we have adopted it as a* standard procedure: a double evaluation, first in fluorescence and then in Papanicolaou coloration. Fluorescence represents the basic technic and most of the completely negative cases are simply verified by a Papanicolaou control.

We found that it was quicker to use both control together instead of Papanicolaou only and consequently the laboratory output can be increased.

We have verified the accuracy of the cytologic diagnoses by fluorescence in comparing 3,000 cases by statistics. These cases emanated from the departments of gynecology and obstetrics of the University or from the "Centre anti-cancéreux" of Lyon. Cytologic diagnoses were controled by clinical and histologic means.

We can see that with some experience the results are identical with fluorescence and the Papanicolaou method and that we could use the first one to separate all negative cases which *represented the great majority* of the specimens.

Dye inducible fluorescence in tumor cells. A. L. Bastos, D. Marques, M. T. Batoreu and M. J. Lanca. Laboratorio de Citologia, Instituto Portugues de Oncologia, Lisbon, Portugal.

Several types of tumor cells observed (including squamous cell carcinoma, adenocarcinoma and lymphoma as well as tumors of experimental animals), growing in vivo or in vitro, show bright fluorescence of cytoplasmic granules, as has been previously reported (Bastos et al., Z. Naturforschg *23b* 969- 1968). This fluorescence is observed in unfixed tumor cells after supravital staining with a particular batch of toluidine blue. Similar fluorescence is given by tumor cells fixed in formalin and stained by Nile Blue sulphate samples from several manufacturers. Both types of fluorescence are excited by blue light (400 nm).

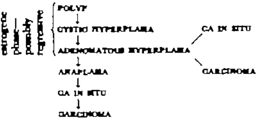
Because supravital staining is simple and observation time short (about one minute) the dye inducible fluorescence (DIF) test may be useful in cancer cytodiagnosis. Hence a blind comparative study of the DIF test and standard stained smears was undertaken on 254 patients with suspicion of cancer. In 27 of the cases results by both methods were not in accord. In the remaining 227 cases the results of the two methods were in agreement. In two cases the DIF test gave positive results while the standard stained smears (and in one case, histology of a lymph node biopsy) were reported as non-malignant. These two cases later developed cancer (an acute leukemia and lymphosarcoma). Difficulty was experienced particularly in two situations. The DIF test showed positive results in severe Trichomonas infestation of the cervix associated with epithelial dysplasia; fluorescence disappeared after treatment for a few weeks. Fluorescence was also seen in DIF tests of mammary duct cells in papillomas (one case with premalignant changes) and we recommend that in such cases cytologic study should be supplemented by histologic examination of biopsy specimens.

In most cases the DIF test gives a valuable pointer to the presence or absence of malignancy. For this reason and because it is easily and quickly carried out, the DIF test deserves careful evaluation as a cancer cytodiagnostic procedure.

Value of the acridine orange method in the detection of the endometrial carcinoma. Z. Singer and D. Šips. Laboratory for Gynecological Cytology and Cytogenetics, Department of Gynecology and Obstetrics, General Hospital "Dr Ozren Novosel" Zagreb, Yugoslavia

Out of 93 cases of hystologically determined endometrial carcinoma we were able to detect 91 per cent by our cytologic methods NEGATIVE 6% SUSPICIOUS 30% POSITIVE 61% 1 mm in diameter Pyrex glass pipettes were used for lavation and aspiration from the uterine cavum. After centrifugation the pallet was separated and used for detection of the cancer cells by the following methods Papanicolaou staining, acridine orange (L. Bertalanffy Science *124* (1956) 1024), rapid sexing and kariotypization (A. R. Sanderson, Lancet *1* (1960) 1252 J. J. Yunis, Human Chromosome Methodology Academic Press N. Y.–London 1965) Acridine orange fluorescent microscopy is an important tool in the detection of endometrial carcinoma for it is not blurred by inflammation degeneration, and high volume of blood specimen.

Table 2 shows hystogenesis of endometrial carcinoma based on prior biopsies and curettage done on patients having endometrial cancer (C. Dallenbach Hellweg, Endometrium, Springer Verlag 1969; A. T. Hertig, Cancer (1919) 916)



It is important to detect when carcinogenesis becomes malignant by cytologic methods. With the acridine orange method it is easy to detect flame-colored cytoplasm of cancer cells, but it is not possible to differentiate carcinoma in situ from invasive endometrial carcinoma and determine the localization of the malignant process. One should try to find methods for evaluation of early cancer of the endometrium or carcinoma in situ in order to confirm positive cytologic findings.

Amniotic cytology. J. E. Jirásek and B. Faltinová. Research Institute for Care of Mother and Child, Prague Czechoslovakia

A

Amniotic fluid provides important information on the fetoplacental unit. The fluid is relatively easy to obtain by amniocenthesis beginning at the 12th–14th week of gestation. The fluid may be analyzed chemically and cytologically. Cellular material is used for cytogenetic cultivation and for a direct cytologic examination.

The smears are stained according to Papanicolaou, the Feulgen reaction for heteropycnotic chromatin (Barr body-heteropycnotic part of X chromosome and for the small heteropycnotic part of Y-chromosome). Different cell types are described.

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Several types of tumor cells observed (includgroup of slightly suspicious smears (our terminology PAP III+) after trypsin application, marked atypical cells were found approximately in one-quarter of the cases; in the rest there were no substantial changes. The changes of negative as well as highly suspicious or positive smears were studied, too. Histologic examination of these cervix lesions was in some cases also performed. It was interesting that also the colposcopic picture showed typical changes in these cases, where cytologic findings became more suspicious.

We presume, on the basis of our present experiences that the trypsin test is in cytology very advantageous, because it helps to evaluate better the above mentioned lesions and to decide further therapy more rapidly (e.g. cone-biopsy) or to only control patients.

III. General Aspects on Carcinoma in situ of the Uterine Cervix and of the Endometrium

Sex chromatin in exfoliated abnormal cervical cells and histology of dysplasia and carcinoma in-situ. H. Nujoks and A. Castano-Almendral. Universitäts-Frauenklinik, Frankfurt/M. Germany

In histologic sections Liska (1961) described a decrease of the incidence of sex chromatin positive cells from normal cervical epithelium to carcinoma in-situ and towards cervical cancer. Sex chromatin studies in exfoliated abnormal cells of vaginal smears were recently reported by Ford and Miles and Atkin. We investigated 8,411 nuclei of exfoliated abnormal cells of 56 cases with severe dysplasia or carcinoma-in-situ. In only 44 per cent of the nuclei a finely granulated chromatin pattern allowed the distinction between sex chromatin-positive and sex chromatin negative cells. In 31 cases these findings were compared with histologic criteria (localization of atypical epithelium, nuclear size, degree of differentiation, mitotic frequency). The following findings became obvious: severe dysplasia and carcinoma-in-situ did not show differences concerning the sex chromatin frequency of exfoliated abnormal cells. Nuclei of cells from carcinoma in situ with early invasion seem to have a lower sex chromatin count. A normal or low sex chromatin count was equally frequent in cases where the atypical epithelium showed small nuclei. Most cases with a low sex chromatin frequency of the exfoliated abnormal cells did not histologically show increased mitotic activity. But there was a tendency that in carcinoma in situ, which show histologically small nuclei and frequent mitotic figures the number of sex chromatin positive nuclei in the smear is lower than in those cases with infrequent mitoses. No correlation existed between the localization of the lesion and the sex chromatin frequency of exfoliated abnormal cells. At present the significance of nuclei with multiple chromocenters is not clear. Only this clarification would allow the determination of the true rate of sex chromatin frequency.

The vaginal bacteriologic smear pattern after coitus with no contraception used. P. Leppäluoto. Laboratory of Cancer Society, Tampere, Finland.

Two hundred cytologic VCE smears taken less than one week after unopposed coitus were studied for the bacteriologic smear pattern and for spermatozoa.

The results are presented, which suggest, that the coccoid bacterial flora could be caused by coitus. The significance of this finding is discussed.

Endometrial exfoliative cytology during prolonged wearing of intrauterine contraceptive devices. F. Havránek and A. Králová. Research Institute for Care of Mother and Child, Prague, Czechoslovakia and Postgraduate Medical Institute, Prague, Czechoslovakia.

Endometrial smears were taken from 270 women with different types of IUDs using them for a period of one to four years. There were 20 nulliparas, 116 primiparas, 102 secundiparas and 52 multiparas. Smears were obtained by aspiration from the endometrial cavity using a polyethylene cannula, diameter 1.5 mm, fitted to a needle with a syringe. Smears were stained according to Papanicolaou and divided into five groups according to the criteria of oncologic cytology.

The total number of smears was 815. 606 (74.3%) of them were valuable. Smears were taken before the introduction of IUD in 188 women, 110 women were examined one year, 155 two years, 63 three years and 18 four years after the introduction of IUDs. Positive findings were not present. Most smears were negative. Only 4.0 per cent out of 606 smears were classified as suspicious. In the first half year after the introduction of IUD the number of suspicious findings increased significantly. The number of suspicious findings two, three and four years later diminished distinctly.

This long term observation of women fitted with IUDs confirmed our previous results based on a short term study that IUDs have no cancerogenic effect on the endometrium.

Cytology of endometrial aspirate during the use of IUD. N. Sağiroğlu and E. Sağiroğlu. Department of Cytopathology and Cancer Research, Hacettepe University Faculty of Medicine, Ankara, Turkey

B

The early prenatal diagnosis of inborn metabolic diseases attracts attention of biochemists, cytologists and cytogeneticists. Suspension of amnionic cells is easy to analyse by histochemical methods. In normal amnionic cells common oxidative enzymes were demonstrated (DPHd, G-6 PDH, LDH and SDH) by means of Nitro-BT. Smears were made on albuminized or gelatinized slides after incubation and after staining with other histochemical methods (neutral lipides, metachromatic granules, P.A.S.), or with conventional cytologic stains.

C

Amnionic degeneration precedes the onset of delivery. Degenerating amnionic cells without nuclei or with pyknotic nuclei are characteristic at term. If such cells are found in the amnionic fluid prior to term a premature delivery may be expected.

Further assessment of the single parameter approach to the diagnosis of a positive cervical smear. R. A. Diamond and T. J. Ryan. Institute of Clinical Pathology and Medical Research, Lidcombe, New South Wales, Australia

Unsuccessful attempts have been made over many years to devise a machine capable of automatically sorting cervical smears into two categories, one to be rejected as negative and the other to be examined by the human eye. With this aim in view and with the Vickers Cytology Screening Apparatus described by Boddington and Diamond (1967) in mind, nuclear size was studied in cervical smears in an attempt to separate the smears into these two categories. The criterion used was a nuclear diameter of twelve microns. A study using this criterion was made of 236 positive smears, 1,300 routine smears and 900 smears from postnatal and postmenopausal women.

The effect of clomid in the biologic picture in endocrinopathies. J. Kobilková, M. Burdová and B. Bodinská. Ist Departm of Obstetrics and Gynecology of Medicine Faculty, Charles University, Prague, Czechoslovakia.

The effect of the non-steroid drug Clomid (clomiphene citrate) on the mechanism of ovulation cannot be explained reliably up to now. It most probably directly effects the eminentia medialis and the hypothalamus anterior (Masoa Carathi). Any evidence of direct effect on hypophysis and ovary, however, is absent.

Vaginal hormonal cytology renders an invaluable service both for endocrinologic research and for evaluation of hormonal drugs. This method is simple and enables one to follow the process dynamically.

As yet the affect of Clomid on vaginal epithelium is contradictory (Widacher, Lamb, Cuderian). Is it perhaps the antiestrogenic effect of Clomid which makes the results contradictory? Therefore 30 women treated with Clomid (5 times 2 pills) for disturbances of the menstrual cycle and simultaneous sterility were studied. Eleven hyperestrogenic women had a Karyopyknotic Index before treatment at least 3 times higher than 6 per cent during the cycle. Thirteen hypoestrogenic women had a Karyopyknotic Index within the limit 32 to 15. Clomid has been administered to 6 women in the menopause who had a Karyopyknotic Index of 0 before Clomid treatment, which means typically atrophic smears. The Karyopyknotic Index in hyperestrogenic women after Clomid treatment decreases strikingly around the 16th day which corresponds to the period of ovulation. A typical progestational effect of progesterone in the smears is missing. In the group of hyperestrogenic women the shape of Karyopyknotic Index curve the same as in hyperestrogenic women, but only in values. The striking break around the 16th day is absent, but there is a striking percentage of navicular epithelial cells, so that this smear has a characteristic pseudopregnancy smear.

After application of Clomid separated cells appear in atrophic smears and the number of intermediate cells is increased.

Now it seems that Clomid in cytologic patterns has some features of an androgenic effect. To some extent there it is a synergist of estrogens (in hypoestrogenic women) and an antagonist of estrogen in those women whose Karyopyknotic Index before administration of Clomid was high.

Trypsin test as the possibility of improving the cytologic diagnosis. V. Tikovsky, J. Kahla, B. Svoboda and V. Kardová. Department of Obstetrics and Gynecology, Charles University, Prague, Czechoslovakia.

The occurrence of doubtful or "slightly suspicious" smears causes the most trouble in cytologic diagnosis. We have used the trypsin test (Kiricuta et al.) in order to try to get better results in diagnosis of these smears.

After colposcopic examination, cytologic smears were taken from the surface of uterine cervix as well as from the endocervical canal. After the trypsin powder 1[illegible] ANS/gr was applied to the cervix and after 4 hours colposcopic and cytologic examinations were repeated. It was found that the smears after trypsin application showed an increase of exfoliated cells, mainly of cells from the deeper layers. In a group of slightly suspicious smears (our terminology PAP III+) after trypsin application, marked atypical cells were found approximately in one-quarter of the cases; in the rest there were no substantial changes. The changes of negative as well as highly suspicious or positive smears were studied, too. Histologic examination of these cervix lesions was in some cases also performed. It was interesting that also the colposcopic picture showed typical changes in these cases, where cytologic findings became more suspicious.

We presume on the basis of our present experiences that the trypsin test is in cytology very advantageous, because it helps to evaluate better the above mentioned lesions and to decide further therapy more rapidly (e.g. cone-biopsy) or to only control patients.

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Cytology of endometrial aspirate during the use of IUD. N. Sağiroğlu and E. Sağiroğlu. Department of Cytopathology and Cancer Research Hacettepe University Faculty of Medicine, Ankara, Turkey

Intrauterine devices (IUD) are being used increasingly in *family planning* all over the world. In spite of considerable work on this subject the role of IUD in contraception and their mode of action as well as the mechanism of temporary sterility in women using the devices were unknown.

The present study has been done by the authors in conjunction with previously published report to elucidate these international problems of medicine and science.

Employing a rather unusual cytologic technique in addition to the new Loopal smear method endometrial cavities of *women*, using Lippes loops from three months to four years, were aspirated when the device was in place. To *do this*, a *sterilized* metal or plastic cannula mounted on a 10 cc syringe was used.

The *material obtained* was *transferred to* glass slides and four smears were prepared from each aspirate. Each of the smears were stained according to Papanicolaou, TMK 101 Turk Toluidin blue and PAS staining methods.

In microscopic examination up to 100 000 macrophages, most of them actively engaged to phagocytosis were observed in each slide. In addition to this, other types of reticulo-endothelial cells, such as fibroblasts, reticulum cells, *monocytes, lymphocytes and mast cells* were also observed.

Macrophages were the dominating cells in the aspirated material. They showed mitotic divisions and phagocytosis of spermatozoa frequently when coitus was performed within 16 hours prior this examination.

From these observations it was also concluded that an IUD acts as a contraceptive through the macrophages, the wandering cells of reticulo-*endothelial system which are stimulated by the* device and accumulate in the uterine cavity. Through the phagocytic and enzymatic action of the macrophages on the spermatozoa and bacteria unwanted pregnancy and some genital *inflammatory* diseases are prevented.

Cytology as a dynamic clinical and research method seems to have solved this important scientific problem.

Results of Papanicolaou smears in pregnant women W Mehring Stadt Krankenhaus, geb.-gyn Abt München Germany

Papanicolaou smears were obtained from all pregnant women of our out patient *department*. Frequency of dubious and positive smears, their *histologic* correlation—when histologic examination was necessary—as well as resulting clinical consequences will be discussed.

Treatment of precancerous lesions of the cervix uteri by vitamin A H G Hillemanns, M Hilgarth and O Fettig, Universitats Frauenklinik, Freiburg Germany

Cyto-histologic research was made on the question of efficiency of vitamin-A-cream on normal and abnormal squamous cells of cervix uteri.

After local application of vitamin-A-cream in a composition of 1% on atrophic squamous epithelium of cervix and vagina of castrated rats, no sign of proliferation or maturation of the epithelium was to be seen. With the animal test, however as well as among the treated women, we noticed inflammation of the vagina and vulva. *The reason must be due to the active* substance of vitamin A because the ground substance alone never showed such symptoms.

Normal squamous cell did not show any alterations except a general irritation of the *epithelium with signs of partial* kolliquation and intensive desquamation of the superficial layers, which often were exfoliated in clusters into the lumen. Basal cell layers were never altered.

The abnormal cell showed enlarged nuclei *with partial condensation and clumping* of the chromatin structure even to the point of pyknosis or karyorrhexis. In cytoplasm, vacuolization and dispersation of structure often were seen. The similarity of these alternations with *those treated with ionizing rays and the* cytotoxic effect of Endoxan and Trenimon, was explicated with little possibility of the vagina reacting upon different noxes.

Treatment of the same patient more than five *times by vitamin-A-cream* was not possible because local compatibility was too bad. Further research with other pharmaceutical preparations of vitamin A will have to be made to give evidence of a therapeutic effect on dysplasias and *carcinoma in situ of the cervix uteri.*

A casuistic report about cytodiagnosis and [illegible] of cilioepithelial tumor cells. M Hilgarth and F J Kaltenbach. *Universitats-Frauenklinik*, Freiburg Germany

This report concerns the rare verification of the cilioepithelial cells, according to Ebner in a Papanicolaou colored smear of ovarian cyst puncture. The histologic clearing up of the extirpated cystically changed ovaries, resulted in benign cystadenofibromata of the ovaries. The consequence for clinical work will be the extirpation of existing ovarian tumors, if cilioepithelial cells can be verified. This step seems to be necessary because of the doubtful dignity of the cells.

Cytohormonal evaluation in the screening of menopausal women A Magát Department of Gynekology and *Obstetrics* of District Hospital Ruzomberok, Czechoslovakia

Cytohormonal screening of the menopausal population was performed because of the survey of the epithelial atypia in the genital organs is, in our circumstances, more preferable than the [illegible] praebioptic methods.

The evaluation of the hormonal situation of these patients was included into the gynecologic examination of all postmenopausal patients. There is an indication of age date of last menstrual bleeding, ectocervical findings, presence of vaginal discharge, and information of personal troubles and changes possibly dealing with the hormonal defect, stated on the accompanying label with the cytology specimen. Two smears from the portio and endocervix and a special one from the vaginal fornix were taken.

The analysis according to clinical evaluation of cytologic changes in the postmenopausal period with respect to the therapy is made.

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BOOK REVIEWS

The Nucleolus By Harris Busch and Karl Smetana. Published by Academic Press, New York, 1970, 626 pp Price $29.50

This interesting book, dedicated to the concept that there is Order in the Universe deals exclusively with the nucleolus, and the nuclear structure essential for synthesis of ribosomes in most cells.

The table of contents encompasses twelve chapters as well as author index and subject index.

The book features a detailed presentation of topics as the relationships of the nucleoli to the overall function of the nucleus, variability of the nucleolus and its substructures in living systems, the cytochemistry and ultrastructure of the nucleolus, nucleolar DNA, RNA, proteins and enzymes. It also covers the nuclear ribonucleoprotein network as an organizational element for transmission of the nucleolar product to the cytoplasm nucleoli of specialized cells, the effects of drugs and other agents on the nucleolus, methods of isolation of nuclei and nucleoli and cytological and cytochemical studies of the nucleolus.

The micrographs and photographs are clearly labelled and reach a level of excellence

The book is well done and should prove of considerable value to graduate students and advanced researchers concerned with new information about the cell

Marluce Bibbo, M D M I A C.

Atlas of Oral Cytology By Herman Medak, Elizabeth A. McGrew Paul Burlakow and Richard W Liecke. Washington, D. C., Public Health Service Publication No 1949 1970 *Price $4 75*

This excellent *Atlas of Oral Cytology* deals with normal and pathological conditions of the oral cavity

The first part of the Atlas concerns the basic cell characteristics of normal epithelial cells originating in different locations of the mouth, connective tissue cells, leukocytes and histiocytes Nuclear changes, cytoplasmic changes and cell groupings are presented in detail.

Non-neoplastic conditions such as inflammation, reaction to dental appliances, and dermatologic conditions are dealt with in the second part, and constitute one of the highlights of this Atlas.

The third section encompasses benign neoplasms, malignant neoplasms, and radiation effects.

The last section is devoted to specific problems in interpretation and difficult decisions.

A descriptive text on selected topics is also presented The color photographs reach a level of excellence and the inclusion of black and white *photomicrographs of histopathologic* material is very opportune and didactic.

This Atlas, therefore, covers a wide range of important aspects in cytology of the oral mucosa, in addition to lesions of the lips and adjacent skin, and is highly recommended.

Marluce Bibbo M.D M.I.A.C.

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News from Societies of Cytology

La Sociedad Española de Citologia

VI CONGRESO NACIONAL DE CITOLOGIA

La Sociedad Española de Citología va a celebrar su VI Congreso Nacional. Los temas oficiales del congreso serán los siguientes

—Citoquímia aplicada a la Ginecología
—Ultraestructura en citología ginecológica
—Citología mamaria

Las condiciones para presentación de trabajos pueden solicitarse, así como cualquier tipo de información al secretario del congreso

Dr Arbolí
Virgen de Loreto 21
SEVILLA, ESPANA

Debe enviarse un resumen no superior a las 250 palabras antes del primero de Mayo de 1971

Han confirmado su participación en calidad de invitados extranjeros los siguientes profesores y doctores Coupez, Maillet y De Brux (Francia) E. Wachtel (Inglaterra) Nieburgs y Bibbo (EE.UU.)

Se han cursado invitaciones a los profesores Schade (Inglaterra) y De Robertis (Argentina), no habiendo confirmado todavía estos últimos su definitiva participación.

La cuota de inscripción es de 1000 pesetas y los acompañantes abonarán 500 pesetas los no afiliados a la Sociedad Española de Citología abonarán 1500 ptas.

Japanese Society of Clinical Cytology

Tenth Autumn Meeting of the Japanese Society of Clinical Cytology will be held on November 13 1971 in Tokyo.

Slide conference and slide seminar will take place in this Meeting and there will be no presentation of proffered papers

Asociacion Mexicana de Citologia Exfoliativa

The annual meeting of the "Asociacion Mexicana de Citologia Exfoliativa" is going to be held in Zacatecas, Zac., Mexico, from October 28 to October 30, 1971

For further details writ to

A.M.C.E.
Niños Heroes" # 151
Mexico, 7 D.F.

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1030 Chemin St. Foy
Quebec 6, P.Q. Canada

Latin-American Society of Cytology

At the meeting of the III Latin American Congress of Citology Brasil was unanimously elected to the Presidency of the Society for the 1970/1973 term. The Board of Directors of the Latin American Society of Citology is now composed of the following members

President	Dulce Castellar (Brasil)
	Av N S Copacabana, 1072/707
	Rio de Janeiro Brasil
Vice President	Hena Cardona de Herrera (Honduras)
Secretary	Nisio Marcondes Fonseca (Brasil)
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	Rua Pacífico dos Santos
	Recife Pernambuco, Brasil
Voters	Patricia Alonso (México)
	Juan Garcia Geismann (Perú)
	Zenon Gonzales (Paraguay)
	Leo Lencioni (Argentina)

The International Academy of Cytology

The Executive *Council of The International Academy of Cytology* for the years 1971–1974 was elected recently The list of Officers and Members of the Executive Council is as follows

President	Emmerich von Haam M D F I A C
	Professor of Pathology
	Ohio State University
	410 West Tenth Street
	Columbus Ohio 43210 U S A
President Elect	Peter Stoll M D F I A C
	Professor of Obstetrics and Gynecology
	Director Frauenklinik
	University of Heidelberg
	Klinikum
	Mannheim, Germany
Past President	*Jorge Campos* R de C M D F I A C
	Professor Department of Morphology
	Facultad de Medicina
	Universidad Nacional Mayor de San Marcos
	Apartado Postal 5492
	Lima, Peru

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ANNOUNCEMENTS

New Orleans Association of Cytotechnologists

May 22–23, 1971
New Orleans Louisiana

The New Orleans Association of Cytotechnologists will present two days of cytology seminars on Saturday May 22 and Sunday May 23 at the Jung Hotel in New Orleans, Louisiana. The topics to be presented will be

(1) Gastric Cytology
Nelson D Holmquist, M.D Department of Pathology LSU Medical Center New Orleans, Louisiana

(2) Cytogenetics
John Holden, M.D The Pathology Laboratory New Orleans, Louisiana

(3) Special Diagnostic Problems in Pulmonary Cytology
Eleanor Bechtold, M.D Department of Pathology Upstate Medical Center Syracuse, New York

(4) Adenocarcinoma of the Female Genital Tract
Soffy Botero, M.D Department of Pathology Charity Hospital, New Orleans, Louisiana

Microscopes will not be necessary as seminars will be built around kodachromes. The fee is $25 00 for cytotechnologists, interns and residents, $75 00 for pathologists, and $5 00 for cytotechnology students. This fee includes a Saturday luncheon For more information and/or application blanks please write New Orleans Association of Cytotechnologists, 6453 Derbyshire Drive, New Orleans, La. 70126. The deadline for applications is April 15 1971 and attendance is limited.

California Association of Cytotechnologists

THE SEVENTH ANNUAL CYTOTECHNOLOGY WORKSHOP-SEMINAR, sponsored by the California Association of Cytotechnologists, will be held on Friday Saturday and Sunday April 30 thru May 2, 1971 at the Master Hosts Inn, San Diego, California. This meeting, held in cooperation with the American Cancer Society California Division, has been consistently regarded as one of the most outstanding programs of its kind, traditionally inviting prominent figures in the field of Cytology as guest faculty

The speakers for this meeting will be Robert R Eggen, M D who will discuss Cytogenetics, and Alan B P Ng, M D who will address himself to the subject of Adenocarcinoma of the Female Genital Tract.

Additional information regarding this program may be obtained by writing the Workshop-Seminar Coordinator California Association of Cytotechnologists, 1127 Wilshire Boulevard, Suite 310, Los Angeles, California 90017 Telephone: (213)481-0300

Other announcements by The International Academy of Cytology

FIFTH INTERNATIONAL CONGRESS OF CYTOLOGY The Fifth International Congress of Cytology will take place in Bal Harbor Miami, Florida, U.S.A. (Hotel Americana) May 29 to June 2, 1974 Details about this Congress will appear in *Acta Cytologica* beginning 1972.

SECOND INTERNATIONAL TUTORIAL ON CLINICAL CYTOLOGY The Second International Tutorial on Clinical Cytology will take place in Vienna, Austria (Vienna Intercontinental Hotel) June 11 to 17 1972. The Tutorial is cosponsored by the Austrian Society of Cytology and the European Federation of Cytology Societies Information about registration and details about the program may be obtained from The Center of Continuing Education (Attn. Mr Claude M Weil) *University of Chicago, 1307 East 60th Street Chicago, Illinois* 60637 U S.A.

Österreichische Gesellschaft für Angewandte Zytologie

In der Zeit vom 4 bis 6. Oktober 1971 findet in Wien eine gemeinsame Tagung der Österreichischen Deutschen und Schweizerischen Gesellschaften für Zytologie statt

Hauptthemen

Zytologie der Lymphknoten Zytologie von Organpunktionen. Einsatz von *modernen Methoden in der praktischen Zytologie und* in der zytologischen Forschung Zytologische Abklatschtechnik. Zytologie unter dem Einfluß ovulationshemmender und ovulationsauslösender Medikamente. Freie Vorträge.

Kongreß-Sekretariat

Dr Rudolf Ulm I Univ Frauenklinik, Spitalgasse 23 A 1090 Wien, Austria

JAMES W. REAGAN, M.D.
Professor of Pathology, Director, Cytology and Surgical Pathology, Institute of Pathology, Case Western Reserve University, Cleveland, Ohio.

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FOURTH TUTORIAL ON CLINICAL CYTOLOGY

August 9 to 21, 1971

Four Separate Special Programs

(1) GYNECOLOGIC CYTOLOGY
August 9–13, 1971

(2) DIAGNOSTIC CYTOLOGY OF OTHER ANATOMIC SITES
August 15–18, 1971

(3) AUTOMATION IN CYTOLOGY
August 18–19, 1971

(4) HUMAN CHROMOSOMES AND CHROMATIN
August 19–21, 1971

The International Academy of Cytology and The American Academy of Reproductive Medicine in cooperation with the Center for Continuing Education of the University of Chicago will sponsor a 12-day course on diagnostic cytology. The course is for pathologists, for residents preparing for the American Board examinations (American Board of Pathology and American Board of Obstetrics and Gynecology), for clinicians interested in diagnostic cytology, for the experienced cytotechnologist and for the cytogeneticist.

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Registrants for the first two sections of the Tutorial are required to bring their own binocular microscopes and light sources. Rental of microscopes may be arranged for a limited number of registrants for a fee of $48.00 per microscope for the first two sections. Microscope rental for the first section alone is $35.00, and $20.00 for the second section only.

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Cytology of Recurrent Carcinoma *Dr Koss*
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Registrants should have their confirmed room reservations six (6) weeks prior to the onset of the Tutorial If not please write or call Mrs. Eleanor OLIVER, Center for Continuing Education, University of Chicago 1307 East 60th Street, Chicago, Illinois 60637 (Tel. No 312—288-2500) As a general rule, accommodations are confirmed at the time of the acceptance of the registration

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Fourth Tutorial on Clinical Cytology

August 9 to 21 1971

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LETTERS TO THE EDITORS

Transthoracic Needle Biopsy for Diagnosis of Lung Tumors

To the Editors:

Transthoracic needle biopsy on the diagnosis of primary or metastatic lung tumors, has been well documented. In the past the method has also been used for bacteriologic studies in pneumonia; however, there is nothing written about the diagnosis by these means on micotic diseases.

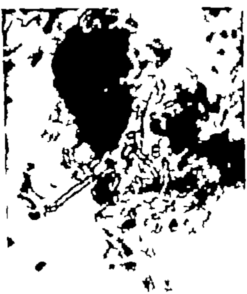

Fig. 1. Minute fragments of hyphae of Aspergillus.

Case Report

A 59 year old, white female entered the General Hospital of Mexico City with the chief complaint of trigeminal neuralgia. Roentgenologically, a 5 cm nodular shadow was discovered on the apex of the left lung. Intradermal reactions to coccidioidin, tuberculin, and histoplasmin were negative, and all the pertinent studies yielded negative results. Transthoracic needle biopsy was performed and necrotic material, inflammatory debris and minute fragments of hyphae consistent with Aspergillus were discovered (Fig. 1). A thoracotomy was done and the surgically removed specimen was a well delimited 5 cm nodule surrounded by lung parenchyma. Cross section revealed a bronchiectatic dilatation containing grass-like material; the cavity was lined by bronchial epithelium and the grass-like material was found to be composed of numerous hyphae of Aspergillus with some fructification (Fig. 2).

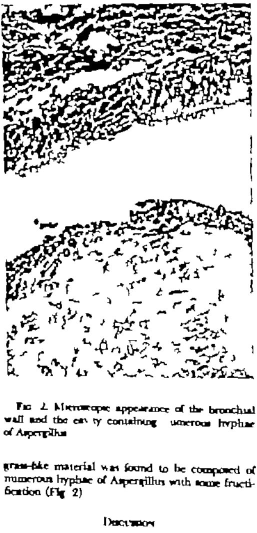

Fig. 2. Microscopic appearance of the bronchial wall and the cavity containing numerous hyphae of Aspergillus.

Discussion

Dahlgren and Stormby have seen similar cases of Aspergillosis diagnosed by aspiration

and contained only few *sperms*. As the smears from the first case were initially screened as possibly malignant an expert opinion was sought from Dr John K. Frost t The Johns Hopkins University School of Medicine. H reported thus, "The eosinophilic-orangeophilic cells appear to contain fatty substance (probably lipoprotein) with pyknotic nuclei. Probably are the lining cells of the spermatocele" (Figs. 1 and 2).

It is known that smears obtained by prostatic massage contain markedly atypical cells with large nuclei. Most uthors agree that these represent cells from the epithelium of the seminal esicles. The spermatocele is lined by cuboidal epithelial cells and presumably this gav rise to the worrisome cells in our fluids. It seems likely that these are normal constituent of spermatocele fluid.

The possibility of spermatic granuloma of the epididymis cannot be ruled out in these men and this would be another possible explanation for the bizarre cells. However after the spermatoceles were aspirated no stony hard masses were felt.

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The Etiology of the Cocci Type Streptokokkentyp Vaginal Smear *

T the Editors

I a symposium in this Journal in 1958, the Streptokokkentyp smear originally described by Wied[1]—and in this *study referred to as the* cocci type smear—was discussed by Gaudefroy and Pundel. The etiology of the smear type was believed to be neither hormonal nor *infectious* but possibly due to lysis of trichomonas. It was suggested that the smear is mostly found in menopausal women or in patients with estrogen deficiency. I the closing remarks the belief was expressed that the streptococci were not the only bacteria in this type of smear and that the exact reason for the smear was not yet understood. In solving the origin of the cocci type smear the present author experience that in this type of smear the spermatozoa are frequently seen, could

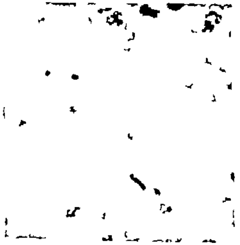

FIG 1 Döderlein type smear Plump bacilli arying in length. The smear "clean" Stripped nucleus as result of cytolytic activity (Original magnification ×1000)

FIG 2 Cocci type smear Small bacteria, either cocci or bacilli covering the epithelial cells Several heads of degenerating spermatozoa isible (Original magnification ×1000)

biopsy. We have the impression that transthoracic needle biopsy is particularly advantageous in the diagnosis of small thoracic shadow

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* Presented at the meeting of the Finnish Society of Clinical Cytology Tampere March 20, 1970

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Atypical Cells in Spermatocele Fluid

To the Editors:

During the last six months we have been requested to examine the cytology of spermatocele fluid from three men. These requests are unusual for us, and so we were surprised to see large bizarre cells with orangeophilic cytoplasm in all three specimens. The nuclei of the cells were hyperchromatic and in some cases angulated (Figs. 1 and 2). As these cells are benign epithelial cells we thought your readers might like to be reminded of this possible diagnostic pitfall.

Fig. 1. Large cell near the center with orangeophilic cytoplasm and large hyperchromatic nucleus. Numerous sperms are also seen. (Papanicolaou stain, ×400)

Fig. Stream of cells similar to those shown in Figure 1 running diagonally from top left to bottom right of the picture. (Papanicolaou stain, ×400).

The three men were aged 49, 51 and 70 years and there was nothing to suggest malignancy of the prostate, penis or testes. They were all in good health and had sought medical advice only because of scrotal swellings. The fluid received in two cases was typically opalescent and contained numerous sperms. In the third case it was clear and amber

TABLE II *The Finding of Spermatozoa in Different Types of Smear in Women Under and Over 45 Years of Age*

	Women under 45			Women over 45		
		Spermatozoa present			Spermatozoa present	
Type of smear	Number of smears	Number of smears	% of smears	Number of smears	Number of smears	% of smears
Döderlein	402	40	10%	97	2	2%
Cocci	159	62	39%	60	12	20%
Mixed	79	12	15%	95	7	7%
Total	640	114	18%	252	21	8%

TABLE III *Proportionate finding of the types of smear in women under and over 45 years*

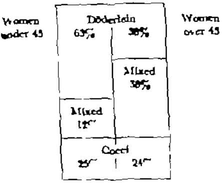
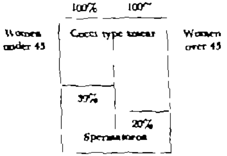


TABLE IV *Proportionate finding of the spermatozoa in the cocci type smear in women under and over 45 years*



of the nonhormonal nature of the cocci type smear (Table III).

The relatively lower finding of spermatozoa in cocci type smears of women over 45 could mean that the type of smear had been established by previous coital exposures, or that the smear is not homogenous entity by composition and/or etiology. Thus the proposed possibility of the cocci type smear being nonhormonal must be accepted with reservation. (Table IV).

The dominating type of smear in the material in which spermatozoa are found is of the cocci type, while that of the whole series was of the Döderlein type. It could be assumed, if the etiology of the cocci type smear were coitus, that the rest of the cocci type smears in the total series actually were caused by coitus, the spermatozoa

TABLE V *Proportionate finding of the types of smear in the total material and in the material with spermatozoa*

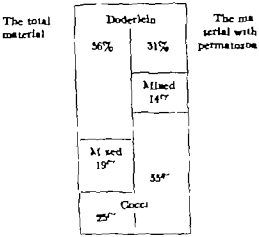
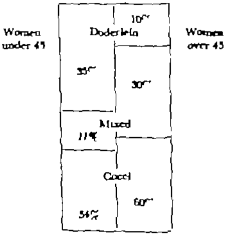


TABLE VI *Proportionate finding of the types of smear from women under and over 45 years in the material with spermatozoa*



prove useful. The frequent finding of the spermatozoa in the cocci type smears seems to suggest that either the spermatozoa are better preserved in this kind of smear or that coitus can produce the smear or both.

Coital etiology seems logical, since more recent investigation has revealed that in the Papanicolaou smear the finding of a few pus cells, a lack of Lactobacilli, and a background of myriads of small bacteria provide a strong clue to the presence of *Haemophilus vaginalis*.[3] On the other hand the *Haemophilus vaginalis* vaginitis has been found to be communicable by sexual contact.[3] The vaginal acidity in cases of *Haemophilus vaginalis* vaginitis has been found to be pH 5.0–5.5[5] and the optimal acidity for the spermatozoa pH 7.0–8.2.[3] This could indicate that the cocci type smear could be the cytologic manifestation of vaginal contamination with *Haemophilus vaginalis* bacteria through coitus.

The finding of spermatozoa in various types of smear led the author to the following study.

MATERIAL AND METHODS

Out of 1000 consecutive Papanicolaou-stained gynecologic smears sent to the author by physicians for routine cancer detection and/or hormonal evaluation, 892 were accepted as representative for the study. The 892 smears were free of trichomonads, a determination of the type of the smear could be made and the age of the patient was known. There were 640 patients under 45 years of age and 252 over 45. Vaginal acidity was reported to the author by one group practice of gynecologists only. The acidity had been tested in 140 cases, using standard pH testing paper immediately prior to the smear being taken.

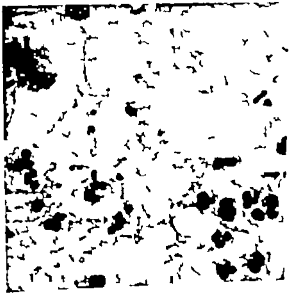


FIG. 3 Mixed type smear. Cocci and bacilli with leucocytes and cellular debris. (Original magnification ×1000)

TABLE I *The finding of spermatozoa in different types of smear*

Type of smear	Number of smears	Spermatozoa present	
		Number of smears	% of smears
Doderlein	499	40	8%
Cocci	219	74	34%
Mixed	174	19	11%
Total	892	133	15%

The types of smear reported here are:

1. The Döderlein type, meaning the finding of a predominance of pale blue, plump, rod-shaped bacteria of variable length in a usually clean smear often with cytolytic activity (Fig. 1).
2. The cocci type smear, meaning the finding of small bacteria, either cocci or bacilli, the bacteria often coating the epithelial cells and appearing in varying quantities in a smear usually having just a few pus cells (Fig. 2).
3. The mixed type, meaning the finding of cocci of various forms with occasional bacilli, in a smear often showing heavy leukocytic infiltration and destruction of the epithelial cells (Fig. 3).

The vaginal posterior fornix specimen was used in reporting the type of smear. The material of the whole slide was used for recording the spermatozoa. Both well preserved spermatozoa and heads of the degenerating ones were reported. All slides were studied by the author under high power (×500) considered sufficient for this kind of study.[4]

RESULTS AND DISCUSSION

The detailed studies performed support the idea that spermatozoa are found more frequently in the cocci type smear. From this finding only it is not possible to conclude whether the high concentration of the spermatozoa indicates that the spermatozoa are best preserved in this type of smear or that the smear is caused by coitus (Table I).

Judging from the presence of spermatozoa, coital frequency in patients over 45 is considerably lower than in the under 45 group. The concentration of spermatozoa, is however greatest in the cocci type smear in both age groups (Table II).

The finding of the cocci type smear with equal frequency in the two age groups with different endocrinologic backgrounds, reflected in different frequencies of the Döderlein and mixed types of smear is in accordance with the proposed concept coital cocci type smear being a physiological condition and the *Haemophilus vaginalis* flora a biological variant of the Döderlein flora should be considered.

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TABLE VII. Vaginal Acidity in the Material with No Spermatozoa and with Spermatozoa

Type of smear	The acidity of the vagina						Total
	With no spermatozoa			With spermatozoa			
	pH 3.5–4	4.5–5.5	6–8	3.5–4	4.5–5.5	6–8	
Döderlein	18	35	14	1	6	3	77
Cocci	0	17	3	1	13	6	42
Mixed	0	12	5	0	2	2	21
Total	18	64	—		23	11	140

only being absent because of contraception or having been autolyzed (Table V).

In the material with spermatozoa, the cocci type smear dominates the picture with practically equal frequency in both age groups, in sharp contrast to the different frequencies of the Döderlein and mixed types of smear. This finding suggests a close adherence of the cocci type smear to coitus (Table VI).

The relatively small amount of material on vaginal acidity shows that the pH of the cocci type smears mainly falls in the range of 4.5–5.5. If representative, this finding supports the theory that the cocci type smear is caused by coitus rather than being a reservoir for the spermatozoa (Table VII).

COMMENTS

This study cannot fully explain why all the smears where spermatozoa are found are not of the cocci type, nor why the spermatozoa are not found in all the cocci type smears. Besides the urethral microbiology of the male partner other coitus-connected factors should be explored to answer the question definitely. The possibility of the cocci type smear not being a homogenous entity by composition and/or etiology should be considered. In addition possible methodic sources of error must be realized: the disturbing role of the diphtheroids[2] and the influence of subjective judgment when determining the type of smear.

The apparently rapid formation of the suggested postcoital cocci type smear, as reflected in the concomitant finding of the spermatozoa and absence of the Döderlein bacilli, makes one question the inflammatory and contagious nature of the cocci type smear suggested by its close identity with the *Haemophilus vaginalis* flora. A more acceptable concept would be that the mechanic, humoral and other possible coitus-connected factors—which we know so little about[4]—could effect an "inborn" change of the type of smear and of the bacterial flora. This possibility is supported by the experience of Amies and Garabedian who have witnessed in cultures, the transformation of Lactobacilli into small coccobacilli, which correspond to the descriptions of *Haemophilus vaginalis*, and vice versa.[1] It is conceivable that the same transformations could take place in the vagina. In this study the transformations mentioned were reflected in the different frequencies of the types of smear in the materials with and without spermatozoa.

CONCLUSIONS

The finding of the spermatozoa in the cocci type smear strongly suggests that the smear is caused by coitus. This suggestion is based on the high concentration of spermatozoa in the cocci type smear of all the material and on the equal dominance of the cocci type smear in the material containing spermatozoa in two endocrinologically different groups of women. The relatively lower frequency of spermatozoa in the cocci type smear of women over 45 compared with those under that age, is suggestive of either a cumulative coital effect or of the heterogenous nature of the cocci type smear. Thus the etiology of the type of smear could also be other than coitus.

The hormonal dependency of the cocci type smear can be neither proved nor disproved by the study. The equal frequency of the cocci type smear in the two endocrinologically different groups of women suggested the smear to be of a nonhormonal nature. On the other hand the proposed heterogenous nature of the cocci type smear does not prohibit the possibility of hormonal dependency.

The suggested formation of the postcoital cocci type smear is postulated as being rapid, and not caused by bacterial contamination but rather by transformation of the Döderlein flora. This was based on the concomitant finding of spermatozoa and absence of Döderlein bacilli in the smear. This could mean that the smear has an indirect hormonal dependency and a noncontagious nature, as the Döderlein bacilli can be found only after hormonal stimulation of the vaginal epithelium.[5] The apparent close adherence of cocci type smears to coitus and to *Haemophilus vaginalis* flora urges the need for further research to explore the basic nature of the smear. The possibility of the proposed post-

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Opening Remarks

Drs Mullaney and Cram and Mr. Trujillo*

Preparation of Samples for Flow Photometers in Automated Cytology

A major aim of an automated cytologic screening system is freeing highly trained personnel from routine screening. The results obtained with an automated system will reflect and be limited by the quality of the cellular material introduced into the system the problems of cell preparation will be the subject of this paper In contrast t the conventional methods of staining cells on slides for microscopic examination, flow systems require that the cells must be stained in suspension (or at least the final state of the sample preparation must be cell suspension) In addition, factors which may degrade the results from an automated system (clumping, doublet cells, cellular and noncellular debris) should be minimized ideally to zero A human observer examining slide can distinguish between the signal (well defined, intact, appropriately stained cell) and the noise (cellular debris, cells stuck together or other undesirable stained objects in the field) machine may have difficulty in making such quality judgment Noise which does not completely obscure the signal may still alter the instrument response. This places rather stringent requirements on sample preparation techniques

In the types of flow photometers developed at this Laboratory [1-3] cells are restricted to 50 μ flow stream where they intersect small diameter laser light beam As each cell crosses this beam, pulse of scattered light and fluorescent pulse are generated under the appropriat conditions of staining and illumination Using modification of the Feulgen reaction[4] with the fluorochrome Auramine-O and the blue light (488 nm) from an argon laser for illumination, DNA distributions for various cell populations have been measured With Chinese hamster cells, the distribution is bimodal. The first peak, of lesser fluorescence, corresponds to diploid cells in G_1, the second to tetraploid cells in G and M In the present system, the instrumentation cannot distinguish between two cells in G which have formed doublet and single G or M cell. It is important, therefore, that cells for this type of photometric measurement be prepared in such manner as to prevent the formation of clumps.

Neither laser illumination nor detector systems are pulsed the detector continuously records the noise arising from any fluorescent material in the sample stream with the cells. If the noise is mainly due to fluorescent particles in suspension, the resulting noise pulses will modulate the signal pulses the signal-to-noise ratio decreases, the shape of the measured fluorescent distribution is distorted, and dispersion in the measurement increases When fluorescent material in solution is responsible for the noise, the dc light level seen by the photomultiplier tube increases and photomultiplier gain shift may occur resulting in increased instrumental dispersion unless the shift stabilizes prior to measurement. It is most important, therefore, that extracellular fluorescent material be avoided in sample preparation. At present this is the major source of noise in the system

Fluorescent Feulgen Staining

Our experience to date with sample preparation has concentrated on adaptation of slide techniques to cells in suspension Most of our effort has been applied to adapting the Feulgen procedure first described by Kasten[4] and later applied by Roch to various types of cells in suspension. Although most of our work has been with Chinese hamster cells, this procedure has also been applied to leukocytes from blood and cells obtained from body fluids (sputum, bronchial tree exfoliation, and vaginal washings) and tissues (lymph nodes, spleen, liver and bone marrow) Because of cell losses in the procedure, it is necessary to start with 3 × 10⁴ cells and minimum of extracellular material. In the case of blood leukocytes, the isolation procedure was adapted from the method of Berger and Childers[5] in which the hite cell buffy coat is treated with mild saponin solution. However in our adaptation the washed buffy coat is resuspended in 10 ml of sequestrene-saline solution to which 3 to 5 drops of 1 per cent saponin solution are added. After standing for 3 to 4 min in an ice bath, the sample is centrifuged t 140 × g Virtually all erythrocytes are lysed in this period The saponin treatment should be minimized both in concentration and time to prevent damage to the white cells (even so, some damage t white blood cells from sick patients may result) Leukocytes in saline retain their integrity for 4 to 6 hours under refrigeration For longer storage, 50 per cent ethanol may be used. Absence of erythrocytes and

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AUTOMATED CYTOLOGY

A Symposium by Correspondence

Chapter V Requirements of Preparation and Sample Collection Problems

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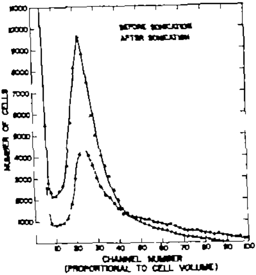

FIG. 2. Coulter volume spectra of Feulgen-stained Chinese hamster cells (●) before sonication and (▲) after sonication. Although sonication effectively eliminated clumps, the debris content has been increased.

Before sonication, the volume spectrum shows some debris (small-volume tail below channel 10). The mode of this distribution is near channel 25. In addition, there are considerable number of larger particles present as indicated by the large-volume tail with the suggestion of second peak near channel 50 (doublets). When this same sample is sonicated, the large-volume tail is eliminated, the number of particles near the mode increases, and the small-volume debris increases. With the exception of the small-volume tail, the sonicated cell spectrum resembles that of normal sample of random cells. Integration under the curve gives measure of cell concentration. Although sonication has increased the cell count 1.3 times by elimination of the high-volume (clump) tail, the peak-to-valley ratio has decreased from 6 to 4 and the debris content has increased 1.6 times. Observation of these same samples in the fluorescent and light microscope verifies that the debris, some of which is fluorescent, is due to cell breakage. In short, sonication will disperse cell clumps but at the expense of some cell disruption. The debris content of the sample is increased, thus increasing the fluorescent noise but not to the extent of obscuring the fluorescent signal.

New Approaches

It would be highly desirable to have technique which avoids pelleting the sample and clumping, thus eliminating the need for sonication. We have recently been experimenting with three alternate methods: filtration through fused capillary array filters, centrifugation through sucrose layers of various densities, and variation of the continuous-flow centrifuge.

Fused Capillary Array Filtration. Fused capillary array filters (Mosaic Fabrications Division, Bendix Corporation) consist of many individual glass capillaries fused together with their axes parallel. Our experiments have been conducted with filters of 3- or 5-μ pore size. The filters are mounted as shown in Figure 3. A slight amount of suction (approximately 1/10 atmosphere) is applied across the filter and the cells are gently agitated by means of Kel-F stirring bar mounted above the filter. This agitation prevents the cells from settling onto the filter surface. The filter is primed with saline, and cells in an appropriate medium are poured in from above. Vacuum is applied and the fluid is removed while the cells are agitated. The filter is not allowed to dry; cells are retained in minimum volume of 5 ml. The next reagent is added and allowed to flow until the previous reagent is flushed out. In this way the steps in the Feulgen procedure are carried out. Cell loss in the fixation step is minimized by use of 4 per cent formalin as fixative rather than the usual methanol-formalin mixture. Nuclear staining is good, debris and clumping are minimal but reliability is poor. Sometimes most of the cells are lost, apparently due to trapping on the filter surface. The released DNA appears to act as binder causing other cells

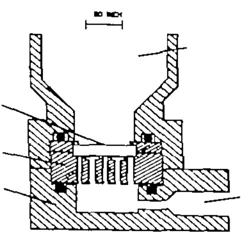

FIG. 3. The glass capillary array filter chamber. The filter (A) is supported in special holder (B) with several holes through it to permit flow. The holder (B) screws into larger chamber (C). Cells are introduced into the column (D). Fluids drawn through the filter by slight suction pass through the exit port (E) to waste bottle. All parts are made of Kel-F.

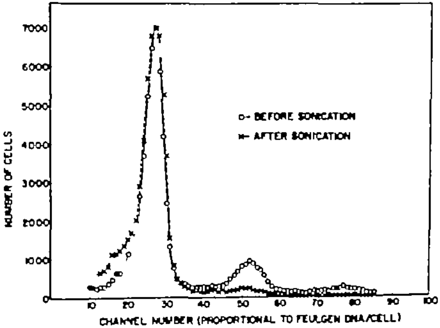

Fig 1 Fluorescent spectra (Feulgen-DNA) of normal human leukocytes showing effectiveness of sonication in eliminating clumps.

integrity of white blood cells are checked by observations of cell volume taken with a Coulter volume spectrometer

Bone marrow specimens may be processed directly by suspension in saline If excessive erythrocytes are present, the above saponin treatment may be applied. Cells from tissue either fresh or alcohol-preserved are prepared by gentle homogenization of a portion of the tissue and allowing the debris to settle Further cleanup may be accomplished by slow-speed centrifugation and or screening through a 35- to 45-μ wire mesh

The fluorescent Feulgen reaction, combined with high-speed microfluorometry provides an automated, rapid method for quantitative determination of the DNA distribution in a cell population. Its potential in automated cytology lies in the ability to characterize normal and abnormal cells in terms of DNA content Our adaptation of Ruch's method allows the entire procedure to be carried out with cells suspended in liquid medium and will not be described here in detail Changes of suspending solutions and various reagents were accomplished by centrifugation and resuspension of the cell pellet Parameters such as time, temperature, solution concentration, normality etc were varied individually over a wide range to produce optimum staining results. Several fixation agents were tried 3 parts methanol to 1 part formalin mixture appears most suitable The entire procedure can be performed at room temperature in the same centrifuge cone (12 ml) within 2 hours using a small clinical centrifuge Centrifugations are performed at 140 × g for unfixed and at 700 × g for fixed cells.

Two major problems were encountered in this procedure cell loss and introduction of cell clumping and debris, which could result in nonuniformity of treatment and also complicate the microfluorometric analysis. Investigating the first problem, a cell count taken before and after the entire procedure indicated a recovery of about 50 per cent. Based on cell counts after individual steps, most of the loss occurs in the early slow-speed centrifugations. In the latter steps, loss appears to be due to adhesion of cells to the walls of the centrifuge cone It is not apparent that this loss is selective of any group of cells within a given population

Cell clumping with the resultant formation of doublets and multiplets distorts a DNA distribution by increasing the size of the G /M peak and introducing spurious peaks beyond it. Shearing during and after the procedure with small orifice pipettes or syringes with small-gauge needles was not effective in dispersing clumps. These may be eliminated by sonication in a Branson S-110 sonifier (10 ml volume, 10 seconds, 40 watts, setting #3) The fluorescent spectrum of normal human leukocytes before and after sonication is shown in Figure 1 The second and third peaks (channels 50 and 75) are virtually eliminated by sonication Some fluorescent debris has been introduced, as evidenced by the growth on the left side of the main fluorescent peak.

The effect of sonication becomes apparent when one views the volume spectra of the same cell sample before and after sonication. In Figure 2 the volume spectra of Chinese hamster cells (Feulgen-stained) before and after sonication are compared.

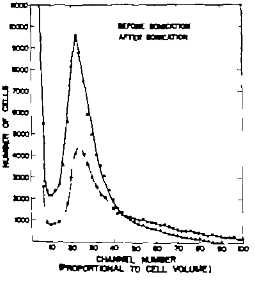

FIG 2 Coulter volume spectra of Feulgen-stained Chinese hamster cells (●) before sonication and (▲) after sonication Although sonication effectively eliminated clumps, the debris content has been increased

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[illegible] Approaches

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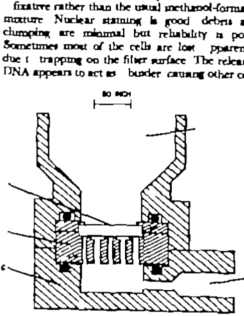

FIG 3. The glass capillary array filter chamber The filter (A) is supported in special holder (B) with several holes through it to permit flow The holder (B) inserts into larger chamber (C) Cells are introduced into the volume (D). Fluids drawn through the filter by slight suction pass through the exit port (E) to waste bottle All parts are made of Kel-F

to clump and stick to the filter thus ruining the preparation. The filters are expensive ($100) and fragile however with proper mounting their lifetime is indefinite.

Density Layer Centrifugation. In this method, the following layers of sucrose in normal saline are placed in a 12-ml centrifuge cone in ascending order 2.5 ml 2.0 M sucrose as a pad *on the bottom,* 0.5 ml 0.55 M sucrose as a collection volume 1.5 ml 0.25 M sucrose as a wash and the cell suspension on top. After centrifugation at 700 × g *for 5 min in a clinical centrifuge* the cells collect in the 0.5 ml 0.55 M collection volume With Chinese hamster cells, recovery is high and debris formation low Volume spectra show little or no clumping and sonication does not change the high-volume tail so that clumping is not serious with this method.

Our initial experiments with Feulgen staining using this technique indicate that the presence of small amounts of sucrose does not interfere with the staining procedure. It may be possible to make up some of the reagents in sucrose and thus to centrifuge the cells through or into an appropriate reagent. This appears feasible based on some very preliminary observations with acridine orange staining of Chinese hamster cells. The cells are removed from the medium and washed with the first centrifugation. Cells in 0.5 ml of the 0 55 M sucrose collection volume are removed fixed in 3 1 methanol formalin and then placed in a second layered centrifuge tube with 2 drops of 0.5 per cent acridine orange mixed in with the 0.25 M wash Fluorescent cells are recovered above the pad after centrifugation. An advantage of this method is that no special equipment is necessary *Recovery appears to be mainly dependent* on the care of the operator and can approach *100 per cent.*

Radial Centrifugation The third method we are trying (suggested to us by Dr N G Anderson of the Oak Ridge National Laboratory) uses centri*fugation not to make a pellet* but to *spread cells* on the cylindrical wall of a spinning container where they can be washed and treated. A glass centrifuge tube (28 mm diameter 40 mm long) *mounts vertically on the drive shaft of a modified* I E.C. clinical centrifuge A lip at the top of the tube provides the necessary holdup volume. The apparatus is shown schematically in Figure 4

Chinese *hamster cells* in F 10 serum are mixed with equal volume of 1 0 M sucrose in the bottom of the centrifuge tube. As the centrifuge is brought up to about 2000 rpm, the suspension moves up the tube wall and forms a uniform layer over the sides of the centrifuge tube. This 0.5 M sucrose solution approximately matches the specific gravity of the cells, slowing down radial migration of cells to the wall *until uniform* layering occurs. After 6 min at 800 × g, 80 per cent of the cells are uniformly spread out on the container wall. When these cells are recovered in normal saline by holding a soft brush against the side of the container as it rotates, they show no change in morphology when observed in the light microscope or with the cell volume spectrometer

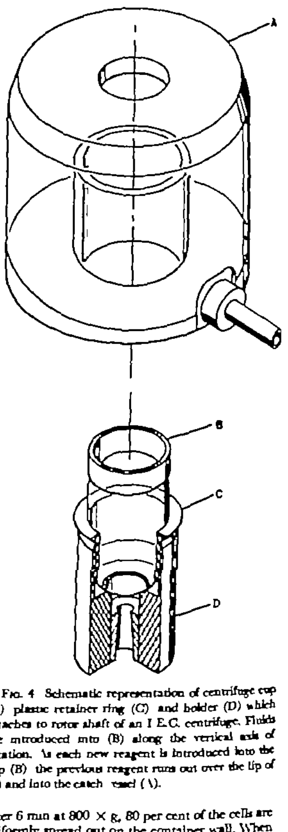

FIG. 4 Schematic representation of centrifuge cup (B) plastic retainer ring (C) and holder (D) which attaches to rotor shaft of an I E.C. centrifuge. Fluids are introduced into (B) along the vertical axis of rotation. As each new reagent is introduced into the cup (B) the previous reagent runs out over the lip of (B) and into the catch vessel (A).

This system can be easily adapted for sample preparation by introducing in sequence at the bottom of the container the solutions normally required for Feulgen staining. Sucrose is added to each successive solution to increase its specific gravity by 5 per cent over the previous solution. The stain or other reagent migrates up the side of the tube, displacing the original solution out the top. This method of changing solutions is satisfactory until the specific gravity of the solution exceeds that of the cells, at which point cell loss becomes critical.

An alternative approach makes use of the radial centrifuge to distribute cells over the wall of the container where they are allowed to air-dry. Once the cells dry and become attached to the glass surface, the container is removed from the centrifuge and passed through the necessary reagents as if it were a glass slide. The problem is how to get these cells back in suspension without disrupting cell integrity and morphology.

Additional Proposals

The preliminary results of density layer centrifugation suggest using zonal centrifugation to establish bands of reagents made up in increasing sucrose concentrations. Staining time, for example, could be controlled by varying the width of the sucrose stain band. After the cells come to rest in 0.55 M sucrose band, a dense, displacing solution pumped to the rotor edge would displace the sucrose bands out of the rotor head. For a discussion of zonal centrifugation, see Anderson. Although a potentially involved procedure, cell clumping and debris formation should be minimized.

Some stains and staining procedures might be compatible with dialysis as a means of changing reagents where the required number of solution changes is small. Acridine orange and ethidium bromide stains are being looked at as possible fluorochromes. Metachromatic staining of cells by acridine orange should enable measurements to be made on DNA/RNA ratios as described by Rigler. Ethidium bromide, under the proper conditions, quantitatively binds to nucleic acids and, upon doing so, increases its fluorescence quantum efficiency by a factor of 20 to 25 as reported by LePecq and Paoletti. A satisfactory procedure for staining whole cells with ethidium bromide has not yet been found.

Summary and Conclusions

At the present time, the application of flow microfluorometry to cytologic examination is limited by problems of cell preparation. The actual fluorescent measurement may be made on the order of 1 min, the preparation time may take several hours. One can visualize an automated cell preparation system based on a filtration or zonal centrifugation method permitting large numbers of samples to be handled at the same time by one operator. Preparations which are clean and free from clumps are essential if such devices are to be used routinely without fear of artifacts arising from the method of preparation. We have outlined our methods, some of the problems, and the avenues we are exploring as solutions. At present, the fluorescent Feulgen procedure is used and cells are removed from each reagent by centrifugation down to a pellet. Of the experimental methods now being tried, modifications of density centrifugation seem most promising.

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DRS. WIED, BAHR, BARTELS AND BIBBO

Preparation Requirements and Sample Collection Problems

It is the purpose of this paper to discuss requirements of sample preparation for automated cytology from the practical clinical standpoint. This approach seems particularly in order since often in the past, practical views and clinical advice were sought only after an automated system design was far advanced in conceptual and engineering development. The clinical sample was often required to be prepared in a specific manner determined by what the design engineers thought would be convenient for their system. Any device, however which is meant to be used in clinical diagnoses must not only be of sound design from an engineering standpoint, but it must pass the most crucial test, namely the test of clinical practicability This practicability is closely if not exclusively connected with the requirements of sample preparation and collection.

Sample requirement for automated cytology may be divided into those related to prescreening which is usually the responsibility of cytotechnologists, and into those related to diagnostic evaluation, which is generally performed by cytopathologists.

Automated Prescreening

Cytologic screening of large numbers of cases became a matter of public health concern only when it became evident that uterine carcinoma as a treacherous disease can be practically eliminated if each woman is annually examined by means of cytopathology It is, therefore appropriate that one concerns oneself in this discussion of automation on cell screening with those problems specifically related to the anatomic site where mass screenings are performed—namely to uterine cancer screening Other anatomic sites have only induced minor public health interest at this time

The use of experimental cell specimens as models in performance tests may be deceiving If one were to state for example that one is able to differentiate Ehrlich ascites tumor cells of mice from human squamous cells of the oral cavity by a machine one could convey the message that one has solved the major problems of machine identification of cells in general, and that one needs merely to apply thus gained experience to a different cell sample and one will have solved the problems at hand in particular Nothing could be further from the truth however The investigator who can differentiate mouse ascites tumor cells from human squamous cells has merely solved this particular problem, but this solution may have only a remote relationship to the clinical screening for uterine or lung cancer

The major problems which have to be overcome are inherent in the preparation of a clinical sample from the female genital tract where heterogeneous cell populations occur It is now a matter of historic record that the ill-fated Cytoanalyzer was built without regard to the sampling problems involved The Cytoanalyzer[3, 4] would have been useful on monolayer epithelial cell smears which were not contaminated with non-epithelial cells, such as leukocytes. The entire project was abandoned because it became evident that no such specimen can be offered in a real world situation.

It should be recognized that the sampling problem is *the* problem in automated cytology Automated analyses could be performed with relative ease if a machine specific sample could be offered to the device Very little glamour is, however associated with research on cell preparation and thus this crucial and mandatory prerequisite of automated cell identification is hardly appreciated.

Other important points for prescreening machines to be considered are

a) ease of sample preparation,
b) economy of sample preparation,
c) possibly required work of duplicate sample preparation,
d) recall system for patients requiring repeat samples.

Ease of Sample Preparation The sample should be taken just as simply as possible. The clinician, who is the one who will take the sample, may defeat *priori* any system too involved or taking more time or effort than the routine Papanicolaou smear It is already difficult to convince the clinician to take separate vaginal, ectocervical and endocervical samples for the cell diagnosis even though this offers hormonal evaluation, assessment of inflammatory reactions microbiologic classification and interpretation of possible irradiation changes in the cell, in addition to the cancer reading It may be a problem to get cooperation from the clinician in regard to getting an adequate sample for automated cytology even if the technic would be as simple to execute as the routine Papanicolaou smear Automated screening offers less than human evaluation It should, therefore be simpler in specimen preparation than the routine Papanicolaou smear

The sample for automated analysis should require less time to take or at worst as much time as the routine Papanicolaou smear It should be as convenient or even more convenient for the patient. It should be possible to simply mail it to the laboratory and should require less handling procedures in the office of the doctor as well as in the laboratory which processes the case eventually

Economy of Sample Collection for Prescreening Most of the economic problems are related to the previous section on ease of sample preparation, since difficulties increase cost It should be clearly stated that any expense for automated prescreening equal to the current complete diagnostic evaluation is unacceptable, let alone the expense being even greater Automated prescreening merely offers cancer screening, thus less than human interpretation which includes hormonal evaluation, microbiologic classification, as well as cancer screening Currently we have difficulties in convincing the clinical staff to use proper fixatives, proper spatula and glass slides for the sample preparation It is often matter of concern to the clinician if one requests procedure which increases the expense by several cents Increases by amounts larger than cents for automated routine screening, which offers less than human screening, are doomed to defeat before they are inaugurated.

Possibly Required Work of Duplicate Sample Preparation Even in the event that the stipulation listed above is fulfilled, it is to be expected that automated screening will increase the total workload rather than decrease it.

To mention practical example Everyone experienced in cytopathology knows that rescreening of prescreened not normal" samples takes more time than reading the slide cold, without prescreening If a slide is prescreened by an individual of lesser experience, the rescreener needs to follow the thinking processes of the less experienced person in order to come to the conclusion that he may overrule the prescreener suspicion, if warranted Had he read the sample without prescreening he might not even have stopped to consider some of the cells which were marked by the less experienced individual A well-trained cytotechnologist will eliminate, to mention an arbitrary figure, about 90 per cent of the cases as normal, and will submit the remaining 10 per cent of the slides for re-assessment to the cytopathologist The cytopathologist has no difficulty checking these specimens since they are on the same slide where the original prescreening was done and where the cells which alerted the cytotechnologist are clearly marked

Unless the prescreening machine can do at least the same, it will do obviously less It will either eliminate less than 90 per cent and/or it will do the prescreening on other than the routine Papanicolaou sample and or it will not clearly identify the cells in question.

If this latter situation is given, duplication is mandatory and all savings in time and energy of everyone involved are lost.

Recall System for Patients Requiring Repeat Samples Presuming that the prescreened sample was read from moving track, rather than on routine Papanicolaou smear and presuming that the machine could eliminate only 80 per cent of the cases as normal, this means that about 60 out of 300 patients will have to be called back for routine smear Fifty-nine of these represent false alarm, assuming that one in 300 patients has cancer If this were so, then it can be expected that the expenses of the recall system, the psychologic trauma to the patient, and the additional work for the clinical staff will vastly exceed any gains made by automated screening of the 80 per cent, even conceding that the machine is always correct in these 80 per cent and that the performance specifications of the machine are so high that it always eliminates 80 per cent of the non-malignant cases in every type of material

Automated Cell Diagnosis

The requirements for cell preparation for a final diagnostic evaluation by machine depend largely on the device which will be used to achieve this task

If automated cell diagnoses are to be used routinely they should be performed on cell preparations which do not require unusual procedures Specimens which can be read only in ultraviolet light, thus requiring quartz slides and quartz coverslips, represent unusual procedures" in this context. Also in this context, staining of the slides with Feulgen reaction, or photographing each cell under consideration and other cumbersome techniques are too "unusual" for routine work Ideally routine slides stained according to Papanicolaou should be usable for automated cell diagnoses However extensive studies on inter and intra-slide variances and on the influences of over or under staining using non-specific staining reactions are necessary before one can determine if routine Papanicolaou stained slides can be used successfully for automated cell diagnoses Studies on these effects are currently underway

As far as TICAS-evaluated cell samples are concerned, it is hoped that routine slides will render themselves for pattern recognition and that the inherent problems of sample preparation can be either eliminated by computer preprocessing and/or by automation of the actual staining procedure to achieve reproducibility in sample preparation

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Discussion

Dr. Adams

The future of automated cytodiagnosis may be with flow systems, particularly due to the speed with which cells in suspension can be handled in such systems. The combination of flow methods for cell handling with fluorescent assay procedures seems especially promising since distributional error (i.e. self absorption error) can be kept quite low

Sample preparation for such equipment can be quite simple, depending of course on the application in mind. Since Mullaney *et al* in previous pages, have well covered the Schiff-staining of cell suspensions, I seek here to comment on some factors which affect the choice of preparation method, and to present an opinion on the advantage of vital fluorescent staining as well as to briefly describe some preparatory procedures which I have found useful.

If we envisage that cytodiagnosis will be accomplished in large regional laboratories, as is now appropriate to Papanicolaou smear evaluation, sample preparation should involve preservation. Given that freezing is cytologically destructive, and that non-freezing low temperatures are difficult to maintain in the mails, there seems no alternative to fixation in this case. On the other hand, if it can be assumed that inexpensive equipment based on flow systems will soon become available, techniques involving the staining and measurement of cells without fixation become practical.

Lasers serve well as illumination sources for high-speed photometry. They can be made stable, and now are becoming quite inexpensive. Recently compact low-power lasers capable of producing intense monochromatic illumination towards the blue end of the visible spectrum have become available. This illumination is capable of exciting fluorescence in many of the stains which have been used or proposed as fluorescent stains. Also it has been recognized that specular photometers based on lasers can serve for cell counting and sizing and also can provide light-scattering data, the practical value of which has not yet been exhaustively evaluated.

Though the value of per-cell DNA analysis in cytodiagnosis is not universally accepted, it is now widely recognized as an interesting parameter. The DNA-specific Schiff reactions are suitable only to fixed cells, and the staining procedure is complicated when applied to cells in suspension. Not all of the fluorescent Schiff reagents decolorize on the addition of sulfurous acid, and thus nonspecific staining tends to be a problem. After acid hydrolysis, cells in suspension tend to aggregate and also, one suspects that cytoplasmic constitution and structure are degraded so that counterstaining or other compound procedures lose efficacy

It would be desirable to have a staining process which does not involve the complexity and the acid degradation of the Schiff reaction, but which is specific for DNA. The europium chelate elaborated by Dyer and Mori[1] seemed to meet the requirement. The stain is easy and fast, has been successful in my hands, and imparts a fluorescence which is excitable with the argon-ion laser. However I have not yet evaluated its specificity

Overall I have been most pleased with acridine orange used as a vital stain. At physiologic pH and osmolarity at concentrations of the order of 10^{-4} and 10^{-7} (w) it is possible to find conditions where the green metachromatic fluorescence follows ploidy peaks quite precisely. Acridine orange concentrations may be found that render the nuclei of formalin-fixed cells green, but stain very little else. In this laboratory we have also determined that, under certain conditions, acridine orange can reliably serve as a vital stain for reticulocytes, imparting a brilliant red fluorescence to the reticulum. Generally at the required acridine orange concentrations, the fluorescence of the solutions is low enough so that the cell fluorescences can be measured in the staining solution.

Cell suspensions can be obtained from a large variety of tissue sources by (1) scraping flushing and straining or (2) mincing and squeezing with saline between two flat surfaces, followed by short settling decanting and straining. Procedure (1) is suitable to the mucosa of body cavities, and also to excised hard solid tumors, and procedure (2) is suitable for soft tissues and tumors. Small aliquots of such suspensions may be added directly to the staining fluid, or if the suspensions are to be fixed, they may be mixed 1 1 with acetate buffered neutral 10% formalin Blood, and other naturally occuring cell suspensions can be added directly to the vital staining fluid

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Drs. Grand nd Frenkel

It is becoming increasingly apparent that automated cytologic prescreening systems will ultimately be designed to examine cells flowing rapidly past an optical sensing system. This approach is particularly essential to the screening of sputum specimens which by their nature tend to present significant sampling problems when handled by slide preparation techniques. It is generally accepted that multiple specimens, each requiring multiple slides, ar necessary to assure high level of accuracy in conventional sputum cytology Extension of this slide multiplicity to an automated system would preclude the possibility of rapid screening Thus, pooling of specimens from several coughs and preparation of uniformly distributed suspension from which representative aliquot can be taken for instrumental examination becomes a necessity for any practical automated cytologic screening system.

Another reason for preferring flow system approach lies in the fact that sputum almost always contain cellular and noncellular debris and clusters of cells, all of which create significant difficulties in instrumental analyses Although some investigators have had limited success in developing computer programs which overcome some of these difficulties, in the long run it is logical to devote one' efforts to the preparation and handling of specimens in way which avoids these difficulties altogether

Much effort has been devoted in the past to the study and development of staining techniques hich are selective and stoichiometric. Unfortunately none of these efforts has resulted in the type of reproducible and accurate staining system desired for an automated system Most of the ork to date has attempted to adapt an instrument to the examination of smears stained with the conventional Papanicolaou stain In view of the complexity of the dy systems involved in this stain and the lack of stoichiometric uptake associated with them, these stained preparations has introduced inaccuracies of considerable consequence to an automated screening system and only slow progress has been achieved to date.

The obvious alternative is to avoid staining by examining the cells in an ultraviolet light system. The utilization of unstained cells has, of course, been considered in the past, but relatively little emphasis was placed upon such studies because of the expense involved in the use of quartz slides and quartz coverslips However once one makes the transition to a flow system, there is no economic reason to avoid the use of unstained cells and every reason to take advantage of the improved accuracy which should be achieved by elimination of the staining process

In conclusion, we feel that where rapid instrumental prescreening is the ultimate objective, specimen preparation forts should be designed to yield uniformly dispersed, well separated, unstained individual cells which can be examined rapidly in flow system. The flow system must be designed to meet the needs of the particular instrumental screening system involved.

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Dr Green

Dr Wied and his colleagues have raised some very important points concerning the clinical practicability of automated cytology devices, and the necessity that the practically realized clinical specimen must be included in the initial system engineering efforts of such device They cite as an example the cytoanalyzer project which was developed around an idealized specimen (from which leukocytes were excluded) with the result that the finished machine could not differentiate carcinoma cells from the leukocytes that invariably occur in real specimen Unfortunately this was discovered so late in development that the whole project had to be scrapped The same fate awaits those who ignore real world problems of specimen preparation in their system conception hoping to delay these unglamorous technical difficulties until the "key pattern recognition problems" have been solved Surely sample preparation is at least one of the problems in automated cytology

However I would like to differ with Dr Wied and his colleagues on several points which they raised concerning the form and function which will characterize an eventual automatic cytology system These points can be categorized roughly as (1) methods used for sample preparation for

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Cell suspensions can be obtained from a large variety of tissue sources by (1) scraping flushing, and straining or (2) mincing and squeezing with saline between two flat surfaces, followed by short settling, decanting and straining Procedure (1) is suitable to the mucosa of body cavities, and also to excised hard solid tumors, and the required analysis of the cervical smear in 5 to 10 minutes and have purchase price in the range of $100,000 If the machine were operated 16 hours a day and the purchase price amortized over four year period, the allocated cost of analysis alone would be $ 35 to $ 70 per patient Also assuming that the machine used $ 30 of consumables (power reagents, floor space, etc.) per sample, and required one-half a technician for supervising its operation ($ 25–$ 50 per sample) the total per patient cost would be between $ 90 and $1.50

Naturally meeting the performance requirement of one analysis each 5 to 10 minutes is the crux of the successful cytology device In my estimation, this is roughly equivalent to one complete analysis of blood film in 1 to 2 minutes Here the cervical smear is considered to be about five times as much work for the machine Although no one has published scheme for performance of either of these tasks in the above specified times, many people are working on the blood film problem, and they consider the speed goals to be reasonable.

This brings us back to the first issue, that of collecting and preparing specimen for such an automated device Without doubt, except for the actual collection of the specimen from the patient, the preparation stream itself will be entirely automated This will not only provide the instrument with much more consistent sample than would be possible with manual methods, but will allow variations of the techniques used today

With this in mind, the issue of costs as they relate to sample preparation can be considered In an automated system there are many ways one might design things that would work However there will be one configuration which is more cost effective than the others It might be possible to design and build machine which would accept as input a randomly selected human collected, human smeared and human stained specimen, and output the correct diagnosis But, even if it were possible to build such device, it would surely be prohibitively expensive For given set of performance specifications, much more reasonable and economical distribution of resources for the hardware would be to develop as an integral part of the system an automated smearing and staining subsystem which could present to the diagnosis subsystem a specimen tailored for a more simple (and less costly) hardware This might also entail slightly different or more complicated procedure for collecting the sample from the patient. Although there might be grumblings from some physicians on changing long established techniques, physicians in the end would acquiesce for economic reasons, if not from the improved care to their patients

It should be obvious from the preceding comments that I consider automatic sample preparation an integral part of automatic cytology Dr Wied and his colleagues are certainly correct that little glamour is associated with work on cell preparation This field has long been neglected, but it cannot remain so indefinitely Where glamour has failed, money certainly will prevail, and in period of decreasing overall funding (both public and private) for research, funds the development of automated clinical laboratory devices are increasing (both public and private) With this stimulus, the long neglected study of automatic specimen preparation should begin to flourish

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DR. HURADN
Re Dr Mullaney et al

The account of sample preparation for flow photometers by Mullaney Trujillo and Crum does highlight the problems of cell loss and increased background noise using fluorescent Feulgen technique, factor of considerable the system, and (2) the ultimate capabilities and *cost of such a device. First, they left the impression that the sample collection, smearing and staining would remain essentially as they are* performed today and that any alternate methods which *were either more time consuming* or more costly than current techniques would be clinically and economically *unviable. Second* they assumed that the eventual automated cytology system would at best *replace a very unintelligent* technician, may make mistaken callbacks, and generally be only marginally useful.

I would like to suggest that these two assumptions may not be entirely correct. Rather than performing as an unintelligent technician, the system that I envision would perform rather like an intelligent senior resident in cytopathology completely bypassing the technician and cytotechnologist and eliminate more like 96 to 98 per cent of negatives. Prescreening and diagnosis would be combined into one automated diagnosis stream, at a cost comparable to what is now charged for routine prescreening. Callbacks would be virtually eliminated or reduced to a low level.

It might seem that this prediction is somewhat optimistic considering the failures of the past and considering that no working model of an automatic cytology device with these performance specifications has been demonstrated. The prediction that such a machine will become *available to the clinical laboratory in the foreseeable* future is based on my knowledge of the *progress which has been made in the automatic* diagnosis of the blood film. Admittedly this is a somewhat *easier problem than the cancer smear* but conceptually it is of the same order of magnitude. Therefore a review of the current state of progress toward an automatic blood film analyzer indicating where the technology would overlap the cancer smear analyzer might help to convince the reader that such an instrument will be available in the foreseeable future.

Basically any automatic laboratory instrument, both blood film and cancer smear analyzer must accept as input a sample taken from the patient process it in some way and output an analysis or diagnosis. What goes on in the black box between *the input and the output* is the subject under discussion. I envision the process *beginning with the automatic preparation* of a smear (or film) of the material to be examined and *automatic staining* of this smear. The smear (or film) will then be scanned by a high speed *vidicon microscope* at one or more wavelengths, and the information digitized and fed into a *computer*-like device. Using a set of location and analysis algorithms, the computer-like device will locate the objects of interest in the field and extract features from these objects. These features will be used to classify each object, and form a diagnosis of the smear. Naturally many microscopic fields must be analyzed before *this diagnosis* can be reached, and the time required to process each smear will depend on the speed at which the computer device-algorithm combination operates.

For an operationally successful device, that is, an instrument which produces the correct analysis *or diagnosis a very high* percentage of the time two of these steps are critical. These are the *automatic sample* preparation, and the algorithms for location and analysis. It is the combination *and interaction of these two factors which will* make or break the performance of any automatic clinical *instrument. The remaining parts of the* process, such as scanning digitization, mechanics, optics, and to some extent, diagnosis, will be adaptations of existing methods and technology. (Naturally the features chosen for analysis must be adequate to specify the diagnosis correctly but this choice I have included in the abovementioned algorithms.)

Much progress has been made developing analysis algorithms for processing the blood film. Several years ago Prewitt and Mendelsohn[4] demonstrated that white blood cells could be separated into four different types using relatively simple means. More recently Young has separated six normal types of white blood cells in the *Wright's stained blood film.* I[2,3] have recently demonstrated that red blood cells in a *Wright's stained blood film can be analyzed* in a similar fashion. Platt Engineering Co and Scientific Product Co. are *marketing devices* which respectively automatically prepare and automatically stain a blood film of high quality and consistency. From these results, it appears that the basic knowledge necessary to build a working blood film analyzer is available.

Where cancer cytology is concerned, this does not appear to be the case, but some progress has been made. Kamentsky's[6] constant flow system showed early promise but has not proved to be as useful as hoped. After many false starts, Dawson, *et al* has *proposed that a successful* machine will use a multiparameter decision space. Wied *et al*[8] has recently demonstrated that two types of tissue culture cells formerly not distinguishable by morphologic or chemical means can be separated by computer evaluation.

For a clinically successful device the additional element of speed and cost must be considered. It is probably easiest to consider these two factors as a price performance ratio that *is for a given* machine price the machine must process enough samples per day to *reduce the per analysis cost* to at least current levels.

Projecting a machine which would perform the required analysis of the cervical smear in 5 to 10 minutes and have a purchase price in the range of $100,000. If the machine were operated 16 hours a day and the purchase price amortized over a four year period, the allocated cost of analysis alone would be $.35 to $.70 per patient. Also assuming that the machine used $.30 of consumables (power, reagents, floor space, etc.) per sample, and required one-half a technician for supervising its operation ($.25–$.50 per sample) the total per patient cost would be between $.90 and $1.50.

Naturally meeting the performance requirement of one analysis each 5 to 10 minutes is the crux of the successful cytology device. In my estimation, this is roughly equivalent to one complete analysis of a blood film in 1 to 2 minutes. Here the cervical smear is considered to be about five times as much work for the machine. Although no one has published a scheme for performance of either of these tasks in the above specified times, many people are working on the blood film problem, and they consider the speed goals to be reasonable.

This brings us back to the first issue, that of collecting and preparing a specimen for such an automated device. Without a doubt, except for the actual collection of the specimen from the patient, the preparation stream itself will be entirely automated. This will not only provide the instrument with a much more consistent sample than would be possible with manual methods, but will allow variations of the techniques used today.

With this in mind, the issue of costs as they relate to sample preparation can be considered. In an automated system there are many ways one might design things that would work. However, there will be one configuration which is more cost effective than the others. It might be possible to design and build a machine which would accept as input a randomly selected human collected, human smeared and human stained specimen, and output the correct diagnosis. But, even if it were possible to build such a device, it would surely be prohibitively expensive. For a given set of performance specifications, a much more reasonable and economical distribution of resources for the hardware would be to develop as an integral part of the system an automated smearing and staining subsystem which would present to the diagnosis subsystem a specimen tailored for its more simple (and less costly) hardware. This might also entail a slightly different or more complicated procedure for collecting the sample from the patient. Although there might be grumblings from some physicians on changing long established techniques, physicians in the end would acquiesce for economic reasons, if not from the improved care to their patients.

It should be obvious from the preceding comments that I consider automatic sample preparation an integral part of automatic cytology. Dr. Wied and his colleagues are certainly correct that little glamour is associated with work on cell preparation. This field has long been neglected, but it cannot remain so indefinitely. Where glamour has failed, money certainly will prevail, and in a period of decreasing overall funding (both public and private) for research, funds for the development of automated clinical laboratory devices are increasing (both public and private). With this stimulus, the long neglected study of automatic specimen preparation should begin to flourish.

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DR. HUSAIN

Re: D. Mullaney et al.

The account of sample preparation for flow photometers by Mullaney, Trujillo and Crum does highlight the problems of cell loss and increased background noise using a fluorescent Feulgen technique, a factor of considerable importance when the principal parameters underlying the detection system are the DNA/non-DNA content of a cell.

Their observations on destruction of cell clumps and the doublets and multiplets formed by sonic and not by shearing stresses, is similar to our experience though this may depend on a time factor and prior fixation of the cell. Considerable disruption of cell clusters occurs by passage through small orifices when using small gauge needles if carried on long enough (i.e. for 3 or 4 minutes) which admittedly is somewhat uneconomic. Sonic and ultrasonic vibrations do disrupt cell links, though prior protective fixation of 4 to 10% formalin or formal alcohol is essential in our experience. The cost is to be balanced against one of cell destruction, and here some form of added enzyme (α chymotrypsin or hyaluronidine or one of the many mucases) may permit of a better balance.

Of their three new approaches the "fused capillary array filtration" appears the least productive and successful for routine use.

The "density layer centrifugation" is intriguing and probably works well for fairly uniform cell samples such as the Chinese hamster cells and blood cells generally. It is more difficult to create proper layering of combinations of cells of ectodermal and mesodermal origin mixed up with mucus and serous exudates. The method is somewhat intricate and time-consuming in schedule, but effective.

"Radial centrifugation" presents the same sort of problems for the mixed cell type sample which may require prior treatment with mucases. The use of soft brushes to remove cells spells further cell destruction and possible cross contamination or the use of costly disposables.

The technique of sequential staining by successive specific gravity sucrose solution is a novel technique and superior to the air-dried method on the side of the tube unless this is on to a removable collodion membrane which can be mounted on a slide or film strip.

Again, with density layer and zonal centrifugation a mixed cell population presents the problems to be overcome. Dialysis staining on the other hand, though slow, has a potential value.

The workers rightly scrutinize the cost and time relationships. Cell preparation, which everyone has come to acknowledge is the most crucial of our problems, could dominate the field in both time and cost, and thus throw automation out on economic grounds alone. This is the one field in which automation should compete favorably.

Re Dr Wied et al.

Drs. Wied, Bahr, Bartels and Bibbo have played the devil's advocate in their critical analysis of the use of automation in cervical cytology. It has taken the world a long time to realize that cell presentation is the key to any automatic scanning system.

The Cytoanalyzer failed for two reasons: firstly, that it had been offered poorly-presented cell material, and secondly that it sought to sift the sheep from the goats in the way of whole specimens. Such is the variance of biologic material that no sensor can extract the relevant true positive signals from such a background—even with the present-day matrix analysis systems.

It thus behoves the creators of automated scanning systems to collect good samples cheaply, then process them simply and cheaply, and then scan them efficiently and also cheaply. Cost is so vital in this field that as Dr Wied and his colleagues aptly state "automation has never really saved money."

General Comments

I have great sympathy with most of the authors' points but would take issue on one or two.

In many fields of well-person screening the cost is phenomenal, as whole populations are involved. In England, the greatest cost to the National Health Service in cytology screening is that of collecting the smear. This costs between 3 and 5 dollars in a well-woman clinic, while the screening costs just over 1 dollar. If a Papanicolaou aspirate pipette is collected, the cost would be about two-thirds that of a scrape smear, and if cytopipette collection by the woman herself is used it costs about half a dollar.

The reliability of the latter technique is less than that of the scrape smear, and in terms of positive signals, i.e. abnormal cells, the normal to abnormal cell ratios are 100:110 for endocervical swabs, 100:35 for spatular scrapes of the cervix and 100:2.5 for vaginal aspirates. The irrigation smear sample is better than the aspirate smear in "abnormal cell rates" (author's observation). The latter two smear samples contain fewer cell clusters, and therefore may rate quite high for automation in spite of their lesser abnormal cell content.

It is well known that if one puts a woman and doctor together they do not part for 15 to 20 minutes and this in itself is expensive. It is correct and ideal that this should occur on certain occasions but screening, to be effective and reliable, may have to be done more frequently, and these intervening screening tests should be economic.

Thus, a nurse-collected or self-collected specimen, though not ideal, may be worthwhile if the alternative is no test at all. Moreover, it has been shown that there is a higher response rate using the cytopipette than a clinic visit for a scrape smear.

Duplicate samples are not so necessary if fixed cell pattern recognition is the form of automation used, as the manual checking can be over the entire tape or slide.

It is here that the choice of stain is vital. As the basis for most scanners is the detection and measurement of DNA of the nucleus, it is essential to compromise between the high signal-to-noise ratio required by the scanner and the more traditional stain for confirmatory evaluation required by the human check screener and cytologist.

Many staining techniques are being tried out. Feulgen is one for the more pure amongst us, but it is not easy to use in bulk processing techniques. The azures, as a variety of stain of cells on solution tracked on film strip, is another approach. Tradition dies hard but Papanicolaou is a complex stain with blue, red, and even green cytoplasmic tone value components. As most workers' aims have been to screen DNA stained with hematoxylin at its peak absorbance value, it follows that simple cytoplasmic staining of a faint variety either green or pink, should give a high signal-to-noise ratio. It will probably require automated scanning over a narrow spectral band while check screening will be by normal white light. By this method a modified Papanicolaou technique or hematoxylin/eosin stain could be usable and directly comparable to glass slide manual screening.

It is only by this partial automation that success can occur in the next few years—until scanners and their memory banks are much improved. Also, the principle of automation will in this way be accepted by the traditional screener as she or he is still involved at a higher and more responsible level than before. At the same time, it would ease the burden of the enormous volume of negative screening, especially in the re-screened groups where the pick-rate is only one or two per 10 000.

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Dr Kline

Preparation requirements for automated cytology appear to fall into two categories. One, how can the specimen be taken from the patient with the greatest ease, and two, how can the specimen be screened by the computer without the usual overlay of cells, or extracellular material as are found in the routine Papanicolaou preparation.

The first problem, as Dr Wied points out, is that the collection of cellular material from the patient must be at least as simple as the routine vaginal smear. In order to obtain single cells, cells should be collected in a fluid medium. Perhaps material obtained by the patient herself with the irrigation smear from the vaginal pipette might be the procedure of choice. In many instances, this material is quite satisfactory. It can be taken by the patient at home without the necessity of an intervening physician or nurse. If it is in fluid form, it might be utilized to pull out single cells or cells that are not surrounded by or superimposed on other cells, in order that measurements may adequately be taken by the computer. In the event that a relatively sparsely covered glass slide becomes feasible to use for automatic screening, it might be stained according to Papanicolaou. Then, if the slide has to be rescreened by the technician and or by the pathologist, no further procedure need be utilized.

Dr Melamed

The two lead papers illustrate two different possible approaches to sample preparation and staining for automated cytologic examinations. Each is related to the kind of instrumentation used.

Dr Wied and his co-workers take maximum advantage of the most widely accepted and most reliable current techniques, using standard Papanicolaou stained slides. Their most difficult problem is not in preparation but in devising an apparatus that can actually examine and measure the cells on these slides within a reasonable period of time, in effect duplicating the performance of the human screener.

Initial efforts have been made by Wied with selected cells or cell populations to translate diagnostic criteria from subjective human terms to objective machine terms. A major advantage in his use of Papanicolaou stained cells on slides is the ease and specificity with which automated and manual methods can be compared on a cell by cell basis. He may be able to build data banks of computer knowledge from these studies that will be useful for classifying normal, abnormal and problem cells. Once that is accomplished, the question of sample preparation must again be assessed, particularly to determine whether accurate final diagnoses can be obtained from detailed analyses of certain selected cells without looking at the total population of cells. It will be interesting for example to learn whether the isolated, single cells that comprise a proportion of the cytologic population in a standard preparation, and can be most easily measured do or do not reliably reflect the presence or absence of precancerous cervical lesions.

Dr Mullaney and his co-workers take the opposite approach. They have discarded the standard methods of specimen collection and preparation, presumably because the Papanicolaou stained cytology slide is not well suited for automated examinations. Instead, they propose to use a flow system of cells in suspension that can easily be examined individually by appropriate instrumentation, and they must necessarily modify the technique of cell collection and preparation to suit the instrument.

They must also devise methods that will enhance characteristic differences between cells in suspension, as they appear to the instrument. This is not necessarily limited to morphologic differences though morphology is important and will undoubtedly remain basic to the diagnosis of cancer

It seems reasonable to begin by adopting standard staining techniques for use with suspensions of cells, without attempting to devise entirely new staining methods. This is what Dr Mullaney and his associates have done They suggest three different ways in which sequential staining with multiple different solutions can be facilitated by filtration, by density centrifugation or by direct flow centrifugation.

A great deal remains to be done with techniques of preparation and staining for cells in suspension, but is only now that they are beginning to be needed. There is good reason to think that this type of methology can develop rapidly

The major advantage in using automated scanning techniques for examining cells on glass slides is that individual cells may be selected as desired to compare or correlate a variety of parameters. Information is obtained for a particular cell or cell type within the total population. The major advantage in using automated techniques for examining cells flowing in suspension is that all cells are examined without selection or bias thus providing information to compare all of the cell types in the total population Each approach has its advantages for particular applications, and each type of instrumentation will require a different method of sample preparation and staining

Dr. Steward

In both of the opening articles mention is made, and essentially confined, to the preparation and handling of dispersed cell samples, whether these be in the form of a smear or as cells which may be serially analyzed in a flow system Also, both are aimed toward the attainment of practically achievable and clinically useful ends Further though the modes and types of analyses described differ widely they are concerned with the pressing problem of automated analysis of large numbers of cells.

However diagnostic cytology can be considered to embrace a wider spectrum of samples and to include tissue sections. Here, although the number of specimens to be handled is usually less than in the examples given, the preparative problems posed are nonetheless important. They may even be more difficult in that the preparative procedure must be concerned not only with proper preservation of the cells per se, but with preservation of cellular and noncellular relationships Certainly the wealth of classical light microscopic observations from which most definitive diagnoses are made, supplies a yardstick by which to judge this phase of analysis Screening techniques have hardly begun in this area, but distinct possibilities exist for their use

Nevertheless, such diagnostic cytology or histology is necessarily concerned with the biopsy specimen which often has its own particular "artefacts" that distinguish it from the autopsy specimen. Further the preparative procedure is one which has to be modulated according to the dictates of the investigation which is to be performed Thus, the routine formalin-fixed preparation may be supplemented by pieces fixed" for electron microscopy and by frozen pieces taken for cytochemical biochemical, or virological study These pose sample collection problems, and to be successful imaginative anticipatory skills and close cooperation of those concerned is a prerequisite Such material, furthermore is usually precious, and the greatest economy in its use is of paramount importance Because of this the difficult decision of whether to pursue a pragmatic approach, or one which aims, albeit empirically for a better understanding of the underlying mechanisms of disorder function is one that has to be made

There is in computer science the expression "garbage in, garbage out " No better exhortation can be made to emphasize the need for investigational materials to be prepared in as optimal" a fashion as possible

What is optimal is a matter of tradition and of evolution, but without it, automated methods or not, will conspicuously fall short of that which is presently attainable

Drs Stewart Van Dilla and Fulwyler

The two very different papers of this section deal with various aspects of the sample collection and preparation problems of automated cytology The paper by Mullaney *et al* Preparation of Samples for Flow Photometers in Automated Cytology describes an experimental attack on specific staining problem, that of adapting the fluorescent Feulgen procedure for cellular DNA to the requirements of high-speed flow system. Their work was primarily aimed at cellular biology research and has been successful in measuring the DNA distribution in tissue culture cell populations, limitations are pointed out and avenues for investigation proposed.

The paper by Wied *et al* Preparation Requirements and Sample Collection Problems, discusses the general problem of sample collection and preparation from traditional, practical, clinical standpoint and concludes that it is virtually hopeless

Our view is that the problem is real and important but amenable to experimental attack and solution—preferably in the framework of large, regional, multiphasic screening (health-testing) centers

The fluorescent Feulgen DNA method described by Mullaney *et al* was developed mainly for flow cytofluorometry of tissue culture cells, although some work has been done with human leukocytes and cells from other tissues These cell samples are clean and homogeneous and relatively problem-free compared with exfoliated cervical cell samples The method is experimental with no attempt at automation It is capable of quantitative DNA staining in the relative sense, that is, cells in G or M contain twice as much fluorescent dye as cells in G_1 The shape of the DNA distribution of cell population in exponential growth agrees with the theoretical prediction based on age distribution The peaks are quite narrow the coefficient of variation of the G peak is about 5 per cent Indications are that dispersion of the distribution can be reduced further by instrumentation refinements The method may or may not be useful for automated prescreening

Wied *et al* emphasize the importance of sample collection and preparation and criticize the tendency of physicians and engineers to ignore it. They point out the need for more research. With this we agree completely but we must disagree with their negative attitude based on assumptions that little or no change from current practice can be tolerated If this view had prevailed some years ago, one wonders whether the Papanicolaou smear technique could have ever been introduced. I may well be that the traditional patient doctor-office visit setting is not conducive to mass prescreening by automated methods. W prefer to view the problem in the context of current trends aimed at much more general delivery of health-testing and screening by multiphasic screening centers. Under these conditions, automated cytology becomes part of a larger program of automated clinical laboratory testing

There are several specific statements and arguments with which w take issue and list below in the order in which they appear

(a) The statement is made that the Cytoanalyzer was defeated by sampling problems that is, it would have been useful on monolayer epithelial cells uncontaminated with non-epithelial cells such as leukocytes. The crux of this issue is not really a sampling problem but inability of the sensor to discriminate between epithelial and non-epithelial cell types normally present. Current trends in cell sensing aim at achieving this degree of discrimination by simultaneously measuring several cellular parameters (for example, cell size, nuclear size, and nuclear DNA)

(b) the sampling problem is *the* problem in automated cytology It seems to us that sampling as currently done is limiting factor but equally important to success are such other factors including adequate discrimination between cell types and high analysis rate

( ) Automated screening ffers less than human evaluation This is really a prediction which may or may not prove to be true. It is also possible for automated screening t yield more than human screening Thus, quantitative cell analysis and data handling may provide real advantages over subjective human evaluation. The frequency distribution of nuclear-to-cytoplasmic ratios is correlated with position in the menstrual cycle and is ffected by birth control pills, patient age, hormonal therapy etc. The frequency distribution of DNA content per cell might indicate early dysplastic and/or malignant change The point is that it is very difficult to make firm predictions about the value of automated prescreening until the best of current designs are built and tested W do feel that the potential is there.

(d) "The sample for automated analysis should require less time to take, or at worst as much time as the routine Papanicolaou smear If the goal is to screen annually all women over 20 years of age, the present system which misses most women would be swamped. Under mass automated cytology program, sampling procedure and time might hav to be modified, but the real goal is to get the job done at an acceptable total cost.

( ) "Automated prescreening merely offers cancer screening, thus less than human interpretation which includes hormonal evaluation, microbiologic classification, as well as cancer screening But cancer screening is the objective, and hormonal status must be taken into account by the machine (as it must by the human) pre screener Microbiologic classification is of lesser importance and not necessarily ruled out.

(f) "Currently we have difficulties in convincing the clinical staff to use proper fixatives, proper spatula and glass slides for the sample preparation." This points out the need for innovation in the direction of a multiphasic health testing program making maximum use of paramedical personnel and standardized mass-testing procedures.

(g) Increases by larger amounts than cents for automated routine screening are doomed to defeat. " We agree that it is desirable to reduce costs. Automation and mass production usually accomplish this However even if one were pessimistic and costs were 25 or 50 cents or even one dollar over present cost (several dollars) it is hard to see why this is fatal Actually cost variations over the United States now are greater than this. In any case we feel that the overriding consideration is to examine all women who need it.

(h) The assumption is made that the patients whose specimens were classified as abnormal by an automated system would have to be recalled for a routine smear But recall would be unnecessary in the flow transport-cell sorting type of automated prescreener because the suspect cells are sorted for subsequent visual examination This approach modified by substitution of pattern-recognition computer analysis for the visual examination, has been already suggested by Dr Wied[1]

(i) The proposal is made that no unusual procedures should be required and that currently used stains and techniques be employed. Again, this is a very conservative attitude tied to the present way of doing things and would be a crippling limitation on the potential abilities of machines. Some innovation may well be necessary if the advantages of cervical cancer screenings are to be extended to all women over 20 years of age.

(j) We feel that machine diagnostic evaluation of the sorted suspect cells is further in the future than automated prescreening but a valuable goal deserving considerable research effort now

Summary

Adequate sampling and proper sample preparation are important for automated cytology (as well as conventional cytology) and present difficulties which we feel are not insurmountable There are other equally important requirements such as adequate instrumental resolution and speed of analysis. Mullaney *et al* describe a Feulgen staining procedure adapted to flow transport systems. Their work was not aimed at cervical cell analysis and, hence, may or may not be applicable. However it represents one of the few attempts to adapt conventional techniques to cells in suspension.

Wied *et al* feel that automated prescreening or diagnosis must be tied very tightly to current procedures and allow little or no innovation. We feel that this is an unduly conservative and pessimistic attitude if the advantages of cancer screening are to be extended to the very large number of women now unscreened. The emergence of multiphasic health-testing centers emphasizing automation of many clinical laboratory procedures would be an ideal environment for use of automated cell analysis systems. For prescreening, we favor the flow transport-cell sorting approach with subsequent visual examination and diagnosis. A parallel research effort at computer assisted pattern-recognition eventually to replace the visual examination and diagnosis procedure should be vigorously pursued

REFERENCE

1 Summary State-of-the-Art Workshop on Automatic Papanicolaou Smear Analysis, conducted by the National Center for Health Services Research and Development, Health Care Technology Program May 8–9 1968 [Copies available from B. D Waxman, Director Health Care Technology Program, National Center for Health Services Research and Development, 5600 Fishers Lane Rockville [illegible] 085.]

MR T[illegible]KO

The two papers offered for discussion differ widely from each other The paper by Mullaney describes his current work and future hopes for an approach using a cell suspension flow system to measure the DNA distribution in a population of cells. There is no mention of a method for the collection of specimens from patients nor is there a clearly stated overall conceptional framework inside which the work is being carried out

Two main difficulties are described the first being the Scylla and Charybdis situation of on the one hand dispersing cell clumps and on the other of simultaneously breaking up the cells and creating debris the second problem is the loss of material during staining maneuvers In an attempt to avoid these two problems a number of more elaborate schemes with other snags are described but one fears that they will be difficult to automate for the preparation of cells for routine screening

The paper by Wied is of a more general nature and is clinically orientated. It states clearly the need to avoid increasing the present work load and cost and points out a number of potential difficulties and misconceptions.

A dilemma is revealed by two statements which occur on page 223 "The requirements for cell preparation for final diagnostic evaluation by machine depend largely on the device which will be used to achieve this task and Ideally routine slides stained according to Papanicolaou should be usable for automated cell diagnoses These underline the difficulty of trying to characterize the requirements for preparation of cytology specimens for automatic screening while being partly ignorant of the nature of the processes to be analyzed and the conditions to be diagnosed. The preparation stage must relate closely to the sensing stage and balance must be struck between them. Not only must the machine specific (Wied page 222) sample be considered but also preparation specific machine must be considered

On the question of cost and possible extra work this document suggests that the labor element is the most important part and that any feasible routine system must be automated so as to avoid an increase in labor requirements and be at least as accurate as present cytotechnicians.

In the summary some tentative principles are listed

a) There must be no loss of diagnostic material during preparation

b) The preparation stage must be an integral part of the complete system.

c) There must be a saving in the work load

d) The system must relate to the patient and clinical reality

e) Full diagnostic evaluation of the specimen must be available as well as cancer diagnosis

f) It will be an advantage to use tried methods and stains if possible

g) It would be preferable to have permanent record which can be interpreted by the human eye.

Closing Remarks

Drs. Mullaney and Cram and M Trujillo

Since several of the contributors to the *Documents* has commented on our paper Sample Preparation for Flow Photometers in Automated Cytology it appears appropriate to clear up several points at this time Our main effort at the Los Alamos Scientific Laboratory has been instrumental (development of various types of flow photometers in connection with the cellular biology research effort here) Because of their availability and widespread use at this laboratory Chinese hamster ovary cells have been selected as the biologic test system for our instrumental developments and preparative procedures Our approaches are experimental and may or may not be directly applicable to specific human samples (e g genital tract specimens) without modifications

We wish to emphasize that we have not yet tried our density-layer centrifugation method with genital material Chinese hamster cells are ideal for our present experiments, since they are nearly spherical and have the same density regardless of position in the life cycle In genital tract material, one would expect several cell types of various sizes, shapes, and densities, as well as debris Variations of these physical parameters will determine the manner in which the cells will "fall" (Stokes law) through the sucrose during centrifugation It may be necessary to modify the density layers or to use an appropriate density gradient to ensure maximum collection of the cells of interest It is possible that some separation between white blood cells and epithelial cells could be achieved Such separation might be advantageous if given machine were confused by white blood cells as was the Cytoanalyzer Although density-layer centrifugation has not been used on samples from the genital tract, the advantages realized on tissue culture cells should carry over to the more difficult samples

The total time required for preparing Feulgen sample using either (a) the sucrose layering technique or (b) ordinary centrifugation is about 2 hours This is determined mainly by the time requirement for the cells remaining in each of the several reagents The time gained by removing the cells from the medium, washing, and fixing in one step with the density-layering technique is about balanced by the time required to prepare the layered centrifuge tubes However many samples can be handled during the same preparation, since 100 per cent of the technician's attention is directed to one sample for only about 25 per cent of the 2-hour preparation time.

One of us (L. S. C.) has experimented with the use of membrane filters for those steps subsequent to fixation with the density-layering centrifugation technique. Morphologic quality and fluorescence specificity do not appear to suffer when compared with centrifugation as means of changing reagents, and there is small saving (30 minutes) of time for the overall procedure

Dr Melamed states in his contribution to the *Documents* Dr Mullaney and his co-workers take the opposite approach They have discarded the standard methods of specimen collection and preparation, presumably because the Papanicolaou stained cytology slide is not well suited for automated examinations. We have not discarded the usual methods of specimen collection we have not considered this aspect of the problem yet It appears that, regardless of the type of automated approach used, genital tract samples will continue to be collected manually in whatever manner provides a sample most suited to dependable diagnosis, regardless of the ease associated with its collection. Apparently we disagree with Dr T S Kline concerning the relative ease of collection Tylko's question of specimen collection from patients is a common problem to be faced by both automated screening proponents and those who favor examining all available cells flowing in suspension.

In general, Dr Husain summarizes our feelings very well radial centrifugation and the fused capillary array filtration have been discontinued in favor of the much superior density-layer centrifugation Dialysis continues to have advantages although slow the ease of automation would eliminate this objection since not much technician time would be required per sample

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DRS WIED BAHR, BARTELS AND BIBBO

Anyone interested in the prospects and progress of automated cytodiagnosis will find in this Chapter an exhaustive review of current views on the general requirements to be fulfilled by any preparation before it enters an automatic prescreener with track flow or fly through. Many more specific comments have been made with respect to each of the systems now being tested or proposed However as we and other experimentalists know numerous additional requirements will be discovered before clinically useful machines can be put to work

One cannot help but be impressed by the generally optimistic tenor of the discussions a sense of I believe is not simply reflecting an ingrained belief in technologic progress, but true vision of real solutions

We wish to direct our initial comments to Dr Green's *Discussion* It certainly would be unwise to forego sample presentation methods more amenable to machine processing provided they retain most pertinent discriminating features, and such diathetic, diagnostic information as might be required We did not, in fact, suggest that a method other than the standard Papanicolaou slide was unacceptable. What was suggested is that other methods would very possibly require a certain duplication of work and increase cost.

There is a certain superficial analogy of blood smear and cervical smear as Dr Green points out It must be understood that successful differential blood counting machines will deal with normal cells only even if their relative proportion is a variable. The machine is far from replacing the hematologist.

And so it is with cells from the uterine cervix Aside from the fact that their natural state is cohesion, but that separation is required, one could envisage a counter for squames, squamous cells parabasal cells and so forth. That machine would not replace a cytopathologist. By the same token, Dr Green's automated intelligent senior resident" is in every respect orders of magnitude away from any blood film analyzer

We find ourselves on common ground with Dr Green's envisioned machine performance i e low recall rate prescreen and diagnostic capability acceptable cost/performance ratio, although considerably less optimistic as far as the financial feasibility is concerned for the next few years Dr Green points out that the subject under discussion is what happens in the black box" between input and diagnostic output, and he expresses his hope that a set of location, feature extraction and analysis algorithms will solve the problem at acceptable cost/performance ratio At this moment these are other expressions for "black box. We do not presently have the data base to decide definitely which features, or image criteria are sufficient and reliable

We also do not, at this time have the hardware components available which would be needed to do the required processing operations efficiently at a cost that would make their incorporation into such a system feasible We have in our data files cell images from several thousand tumor cells, and have spent a concerted effort for the past three years to find image properties (or linear combinations thereof) which yield high likelihoods that a tumor cell is indeed being observed The results give rise to much optimism as far as the feasibility is concerned, but at this moment the processing is much too expensive Feature extraction of discriminating image properties only and classification for 100 cell images requires approximately 15 seconds, with memory requirements of 128K, and at a cost of 15 dollars per minute This time estimate does not include time required for scanning the cells or locating them

Now it is certainly true that once it is known which feature should be extracted, special hardware hybrid components could be designed to bring about a drastic reduction in processing cost and time requirement, also, a final machine would use software written in the most efficient way However we at present, still do not yet know which image criteria make the cytologist recognize the tumor cell as such

Dr Green refers to Dr Mendelsohn's paper on leukocyte identification. This pioneering work was not, to our impression, intended to suggest that the problem of white blood cell recognition had been solved It was feasibility study conducted on 22 cells of four different cell types As matter of fact, the sample size for eosinophils with two cells, and attaching confidence limits even only in three-space on sample size of two, was certainly not intended by the authors as delineation of the pattern space but as a methodologic point of order The enormous effort spent since to develop clinically feasible device was inspired by this work, and herein lies its significance, but it also indicates the gap that separates feasibility study from practical, financially *feasible field device*, in much more simple situation, where the risk factors are small as compared to those encountered cancer prescreening

We would agree entirely with Dr Green as to aims and hoped-for performance of such systems, and the expected cost reduction computing power during the next few years will allow more optimistic outlook at the economic feasibility

Drs Grand and Finkel are unnecessarily pessimistic about selective and stoichiometric staining techniques Feulgen staining and its variants as well as the gallocyanin-chromalum technique can be handled by relatively simple, automated procedures Mullaney and colleagues give an example for fluorochromes which are even simpler to apply Although ultraviolet techniques have distinct merit in the measurement of cell properties, they are beset with their own set of errors to which long history of papers of two decades up to 1960 bears testimony

Dr Husain points about the pressing need for relief from the tedium of uneventful mass screening well taken, if any nationwide screening as public health effort is contemplated

A second good point brought out by Dr Husain that we should not relax our requirements of performance below the level of better cytology laboratories

Dr Mullaney *et al* presented very good results in cell preparations using density layer centrifugation Considering the requirements of this method from the practical standpoint, we would like to ask two questions from Dr Mullaney

1) Is there any way to diminish the time for cell preparation and if so, to which degree might the quality suffer?

2) Do the authors intend to test, or did they test the system for other samples, as for example material from the genital tract? What would the difficulties be with clinical material other than the type of cell population used by the authors

To Dr Mullaney and collaborators and others interested in the suspension of single cells, we suggest—perhaps redundantly— that the process of breaking up cells may be promoted significantly by the combined action of trypsin in low concentration and a calcium chelating agent such as 1 to 3 millimolar Versene Calcium is crucial intercellular ligant, the removal of which alone induces liver cells to separate and has further profound effect on the loosening of intercellular connections in monolayer culture as seen under scanning electron microscope.

We do not agree with Dr Steward that tissue sections could be more difficult to prepare than the cell samples needed for fast automatic analysis. Decades of light and electron microscopy have given us the finest fixing and sectioning techniques ever The problem would be with the time required for the procedures of embedding and sectioning The heart of the problem with tissue is really the fact that rational automated tissue analysis, even in limited areas of pathology will remain dream for many years to come. The "optimal" method of cell or tissue collection and processing will most probably not be left to tradition and evolution as Dr Steward suggests, but will be determinedly sought in planned experiments

Drs Stewart, Van Dilla and Fulwyler have expressed understandable concern about the conservative stance taken by us The role of an *advocatus diaboli* as Dr Husain calls it, was assumed at time when first experiments with prototype machines and rumors of ongoing industrial research in this area abounded Many inquiries from organizations, even countries, were received asking where an automatic system could be purchased

In consideration of this situation, emphatic warnings should be sounded and discussions initiated in order to define real requirements and explore possibilities to the best of current understanding The open forum of this Symposium by Correspondence will no doubt constitute representative survey of facts, thoughts and ideas on automation in cytopathology *Continued* conversation on the part of some participants is a desirable safeguard, and should in no way be understood as an impediment to progress

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Chapter VI Approaches to Prescreening

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Opening Remarks

Drs Hulett and Herzenberg

Special Consideration of Flow Systems

Flow systems make individual observations on cells as they flow sequentially through small detection volume They can observe large numbers of cells in short time periods, typically hundreds to thousands per second, and thus lend themselves to rapid automatic prescreening procedures Common parameters used are light absorption, fluorescence or scatter or electrical resistance of the observed particles Absorption and fluorescence measurements are made with appropriate optical systems and photodetectors, using filters as required to emphasize the selected wavelengths. Fluorescence measurements are often much more sensitive than absorption, since sensitivity is determined by the signal to noise ratio under the experimental condition, and background noise is much lower in fluorescent system than in one relying on absorption. Light scatter is used to measure size using the proportionality between size and light scattering[6] Resistance changes in the detector volume as the cells flow through can also be correlated with size[1] In many cases it will be desirable to use a combination of two or more parameters to characterize a given cell population more exactly

One of the first applications of such prescreening was reported by Kamentsky *et al.*[4] who used a dual optical system to measure the ratio of 2557 nm absorption (due to nucleic acid) to light scatter (a function of cell size) They found cancer cell suspensions generally had some cells with a higher value for the ratio than did benign cell preparations and separated a few such cells from a large population for subsequent visual examination.

Our work on prescreening and sorting has permitted sorting of 10^7 to 10 spleen cells on the basis of fluorescence developed by incubation with fluorescein diacetate (FDA) using spleen cells from immunized animals Cells producing specific antibodies were found to be considerably enriched in the more fluorescent cells and depleted in the less fluorescent cells[2] We have also shown that it is possible to detect relatively small histocompatibility differences between cells and sera, using FDA cytotoxicity tests, a "prescreening" procedure which should be of great value in transplantation work.[3]

Differential white cell counting seems particularly well suited for prescreening by flow systems. Staining characteristics with acridine orange, which preferentially stains anionic groups like nucleic acids and mucopolysaccharides, can serve to illustrate the possibilities of the method Rigler[7] and West have reported that granulocytes and lymphocytes have different fluorescent spectra after acridine orange staining with the latter exhibiting high fluorescence at wavelengths above about 600 nm relative to that below 550 Thus a measure of the ratio of the fluorescent output in the two bands should serve to classify them.

One small subclass of granulocytes the basophils, probably do not follow this rule Because of their high heparin content they might be expected to contain red fluorescing cytoplasmic particles and such particles were observed by West in some granulocytes. The ratio would, in fact, probably be so high for these cells that it would serve to identify them as a separate class Even if this is not the case the lymphocyte-granulocyte ratio would not be seriously affected since the basophil count is only about one-half per cent of the total count.

Other parameters which could be used to identify other subclasses of leukocytes include use of phloxine as a specific stain for eosinophils, and use of a combination of size and acridine orange fluorescence as a rough indication of monocyte concentrations.

Further suggested prescreening applications for flow systems include separation of tumor cells or the Reed-Sternberg cells of Hodgkin's disease from normal cells, either in blood or lymph samples or in tissue cell suspensions, and differentiation and separation of leukocytes with varying contents of alkaline phosphatase which may permit automated differentiation of myeloid leukemia from leukemoid states In fact, any pathologic conditions characterized by changes in enzyme activity in carbohydrate or lipid content, or in any parameter which is stain sensitive or alters a physically measurable quantity can in theory be measured quickly and accurately by flow techniques The practical applicability will depend primarily on the sensitivity of the detector under the experimental conditions.

The methods of preparing the cells for these systems are different from those customarily used because the cells must remain in suspension throughout the analysis While much work remains to be done in this area the problems are comparable to those already solved for fixing and staining cells on slides.

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DR. KAMENTSKY

Special Consideration of Flow Systems

In the design of a prescreening instrument
appears reasonable for the engineer to build
machine which mimics the cytotechnician. Com

puters are now available which can recognize" patterns and surely these same theoretical pattern recognition methods can be employed in the design of machine to scan slides and to identify cells by the same criteria developed and successfully used by the cytologist. I would like to examine this concept in these opening remarks. I will describe why were led to both rejecting the slide as a means of cell presentation to machine, and the classification of cells by morphology alone using pattern recognition techniques.

To characterize cell morphology by pattern recognition techniques, an optical scanner is required to convert the image of cell into sequence of electrical signals. Many different cell scanners has been developed during the last 20 years. In each case the cellular image is represented by a set of values describing some optical properties at discrete points on the cell image. Each cell is usually described by 1,000 to 10,000 numbers with each number representing the optical density at fixed wavelength of different image points. I believe that studies involving high resolution scanning may someday yield fundamental cytologic information, are important to many research uses, and may even provide means of judging difficult cells. However, the direct application of this technique to a clinically useful prescreening device where large numbers of cells must be processed in a few minutes presents serious problems.

A practical prescreening device may be required to analyze 100 to 1000 cells each second if samples of 10,000 to 1 000 000 cells are to be processed in few minutes. This means that the scanner and the accompanying computer must generate and process 100,000 to 10,000 000 numbers each second at each wavelength if each cell is represented by 1000 to 10 000 optical density values.

The signal-to-noise ratio of any scanning device determines the number of meaningful levels of optical density that can represent each image element. This signal-to-noise ratio is physically limited by the number of light photons per measurement. The number of photons per measurement directly proportional to the product of the intensity of the scanning spot and the time that that spot dwells on each image element. We have found that practical scanning systems may be severely limited signal-to-noise ratio by light limitations at scanning rates above 1 000 000 elements per second measuring only two levels of optical density and single wavelength range.

Of greater concern is the capability of the data analysis unit which must process the scan data. Each of the data values from the scanner may have to be manipulated many times to carry out pattern recognition algorithms. Special purpose integrated circuit arrays must be developed for morphologic pattern analysis since the processing rates of even the largest computers will be too slow and certainly too costly for clinical use.

Nearly all of the high resolution scanning systems have used cell preparations on glass (or quartz) slides. Very accurate measurements of single cells have been obtained with scanning devices using manual centering but there has never been clinically usable device that automatically scans unselected cells in large numbers by systematically examining the entire slide. One of the principal problems in designing such a device is that there has been no practical way to isolate cells from each other as they are transferred to slide. This is a major difficulty with epithelial cells that are rubbed or scraped from the cervix and dislodged in plaques or fragments. The normal cohesion of epithelial cells and surface tension factors in smearing cells on a slide contribute to the clumping. Automatic centering and isolation devices that have been used in scanners for print reading may reduce these problems, but there will have to be modifications in cell sampling methods and slide preparation as well.

There are a number of other problems in automatically scanning cells on slide. Boundaries of the microscopic field will intersect cells as the slide is scanned, and cell image reconstruction over multiple fields must be considered. Focus is critical, and changing focus may be necessary to compensate for variations in slide thickness or cell placement or thickness. Electronic control of focus is theoretically possible if necessary but must be developed. Variations in cell fixation and staining or background staining may introduce some problems, but automatic staining devices are available that should be able to minimize these. In any event, a highly accurate mechanized stage is necessary to properly center and move the slide during the scan without disturbing the focus.

Some of the disadvantages of the slide have been circumvented by use of transparent tape to carry the cells. The sample collected in the usual fashion is fixed and a thin line of suspension is "graphed" along the center of a transparent tape for a length of 60–100 cm, corresponding to the area on a normal microscopic slide. The cells are then stained automatically by passing the tape through an appropriate series of staining solutions. There is said to be less clumping than with direct smear preparations on slides and a more even distribution of cells. The full width of the track can be contained within the diameter of the microscopic field, so there is no problem with cell images being transected by the field margin. The entire sample is easily scanned by moving the tape from one end to the other. Abnormal or unusual cells can be located for re-examination later by marking the tape. So far there is little experience with tape transport of cells, and it is not known how much of a problem there will be with variation in focus, cell overlap, background irregularities, resolution, etc.

By increasing the size of the scanning spot until *it is larger in area than any cell*, resolution is reduced to zero. Cells that intersect the scan spot will absorb or scatter the incident energy at specific *wavelengths and scatter angles according to their* cellular constituents and according to the stains that have been used, if any. In appropriate fluorescent or dark field systems they will cause an increase in light. With this method, cell identification is based primarily on relative differences in the mass of multiple constituents of the cell, and on the size of the cell itself; to a lesser extent, it is determined also by the distribution of constituents within the cell, the physical state of subcellular components (granularity), and the configuration of the cell or nucleus.

There have been extensive spectrophotometric and chemical quantitative analyses to characterize differences in the mass of certain cell constituents, and these data should be very useful in establishing criteria for cell identification. There are a number of very practical physical advantages in the zero resolution microspectrophotometric method. First, there is no problem with focus. The depth of field of an imaging system is a function of the required resolution. With zero resolution, collimated light can be used, and there is a large depth of field. The optics and mechanical assembly therefore can be simple and inexpensive.

Without a need to maintain focus, the position of the cells along the scanning beam is not critical, and the problem of cell transport is greatly simplified. There is no need for automatic focusing devices. Either the slide or tape transport system can be used with zero resolution measurements, but it is also uniquely possible to use a liquid transport system with cells in suspension that are examined as they flow through a narrow channel. This is a much simpler means of cell transport than any of the others and useful in that it inherently facilitates isolation of cells during measurement.

Because the scanning spot is large and encompasses the entire cell, the energy available for each measurement is increased by a factor that may be of the order of 1 000 to 10 000 times as great as in high resolution systems. The number of cells per second that are examined can be increased correspondingly and still yield high *signal-to-noise* ratios with cell measurement rates in excess of 1 000 cells per second.

Data acquisition rates are inherently slower than with the high resolution systems, and data handling is much easier. With the system we used, for example, simultaneous measurements were made at four different wavelengths on each cell and cells were examined at a rate in excess of 1 000 per second. The information rate of about 3×10^4 bits/second was conveniently processed by special purpose circuits or a small scale computer (IBM 1130).

There are also biological advantages to the zero resolution system with fluid transport. For example, unfixed cells that are unstained or stained with vital stains, or living cells suspended in a fluid medium that will sustain viability, can be examined. The fluid transport system facilitates cell separation, which can be carried out according to the spectrophotometric properties of the cells. Thus, further examinations of a selected cell population are possible. Methods of sorting cells using a fluid transport are described in another paper in this symposium.

A rapid multiple wavelength cell spectrophotometer using a flow system has been used in a variety of different studies. Unstained cells have been examined to compare absorption at 2537 Å as an estimate of total cellular nucleic acid versus scatter at 4100 Å as an estimate of cell mass. Using the same parameters to measure unstained cells from the human uterine cervix and vagina, Koenig evaluated this instrument as a screening device for cervical cancer detection.[6] Kamentsky and Melamed[7] have described how an instrument was used to differentiate cell populations by means of multiple measurements of absorption, scatter and fluorescence of stained cells. A study of the capability of this instrument in characterizing normal homogeneous populations, as well as tumor cell populations, is now in preparation.[8] Van Dilla[9] has described a single parameter flow system for measuring the fluorescence of cells in suspension stained by a fluorescent Feulgen procedure.

Our current work is involved with developing new property sensor systems to provide morphologic measurements in a flow transport system. We are presently developing a new instrument to provide simultaneous determinations of multiple cellular constituents as well as specific morphologic measures. It is hoped that a family of instruments will be developed from these prototypes with a range of design specifications suitable for carrying out many of the medical laboratory tests of cellular specimens.

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DR. HEMSLEY

Special Consideration of Track Transport

The different requirements of men and machines with respect to the presentation of material for screening is basic problem There is not complete agreement about the nature of the material to be screened which will allow the immediate solution to the problem of building screening machines Ideal prescreening should include no loss of diagnostic information, concentration of information and cheapening of the diagnostic process

Origin of Prescreening and its Relation to Machines

Prescreening arose out of the use of technicians partly as result of there being too few medically trained cytologists to screen all the cytology samples The introduction of technicians can be seen as an attempt to bridge the gap between what is possible and what is actually being done The use of technicians is encouraged by the fact that while they probably make as good decisions as doctors on cytologic material they do not require the same wider based training nor such specialized qualifications to enter for training Because of this technicians can be drawn from wider population than doctors and are both quicker and cheaper to train The assumption is made that the trained technician is competent sort normal samples from abnormal ones and pass an opinion on the abnormality Indeed the technician may be more skilled at his task than the medical cytologist as he devotes all his time and skill to the one task

The concepts of machine prescreening in cytology automation has been influenced by desire to imitate the bov practice a belief that it is possible to cheapen automation by making it two part process and belief that the final decision diagnosis will have to be made by doctor or technician, not necessarily because the machine cannot make as valid a decision as man but for legal or ethical reasons

Prescreening and the Nature of Cytology

In view of the suggestion that all life processes are cybernetic and involve complex feedback some of the attempts at and discussion of prescreening appear to be based on oversimplifications It is assumed that by measuring particular parameter or few parameters it is possible to reliably discover samples having particular type of diagnostic significance The following points should be considered 1) There is as yet no table of quantitative parameters that will allow an unambiguous description, and an understanding of the significance of the various objects and groups of objects that occur in cytology 2) It is just as important to identify and recognize normal samples as abnormal ones 3) The decisions taken at the prescreening phase are as important to the patient as any other decisions if not more so 4) In the attempt to prescreen for *one* condition one may ignore other diagnostic information

There is tendency to ignore the essential unity of all screening and to try and create two classes of samples namely those only prescreened and those also finally screened The attempt to use an incomplete machine as a prescreen leads to two misunderstandings one that man is directly responsible for all the decisions which is not necessarily true for those samples passed as normal by the machine and secondly that machine can be made predictable while only knowing part of complex situation However if sufficient description of cytologic material was gathered and made available then it might be possible from position of knowledge to design and build prescreening machine which while making valid predictable decisions does not possess all the facets of a full analyzing machine

Prescreening and Screening by Man and Machine

There are various combinations of prescreening and screening Where prescreening implies the setting aside of some samples without further study the prescreening and screening functions are combined for the set aside samples The combinations listed here give framework in which prescreening can be considered.

1 *Prescreening and Screening by Man*

This is the only practical system today and should not be neglected as subject for automation. A means of improving a man's output would have an immediate tangible benefit

2 *Prescreening by Machine and Screening by Man*

Once there is a sufficient knowledge of the subject of cytology this combination may be a feasibility In the meantime its apparent simplicity and apparent avoidance of ethical difficulties will cause it to be a much advocated and studied combination

3 *Prescreening and Screening by Machine*

The use of a machine to prescreen for another machine can be envisaged in various ways. A prescreening machine sensing objects in suspension could be followed by a screening machine sensing object on a solid substrate. A number of prescreening machines situated at a distance could select objects or samples which a central screening machine would analyze in greater detail.

The prescreening and screening machine are most likely to merge as integral parts of a multilevel and multiparameter sensing machine where not all the objects or samples are sensed for all the parameters but where the measurements by the various parts of the machine are used to program each other

4 *Prescreening by Man and Screening by Machine*

A machine could be used to make measurements and decisions on material that was not classifiable by man In this situation an expensive but comprehensive machine could be exploited to gain new knowledge and understanding of cytology

Situations analogous to prescreening would occur with a diagnostic screening machine Such a machine would require feedback and interaction with cytologists especially during its initial analysis of unclassified objects, when being used for study and research by cytologists, and on the discovery of new hitherto unclassified objects

Preparation and Presentation for Prescreening

There are two main methods proposed for prescreening The one requires that material in suspension is analyzed while it passes through a tube hole or channel the other method analyses the material after it has been applied to a solid substrate such as a slide or pliable film.

The suspension method allows certain estimations to be made with elegance and speed and there are means for concentrating selected parts of the sample for final screening such as aspirating part of the sample through a filter There are difficulties in obtaining a one to one correspondence between the prescreened object and the objects presented for final screening on a filter The sample is also changed each time it is put through the process either by mixing or by loss of some of its constituents, e g., by aspiration for monitoring By permanent application of the sample to a solid substrate literal concentration of selected objects is prevented short of cutting up the substrate. The onus is therefore placed on location and recall of the object in question. However both the prescreening and screening are performed on the same unaltered object, there is a readily recognizable one to one correspondence between measurements and object and the original sample can be recalled many times without alteration or loss.

Both the suspension and solid substrate methods are open to the difficulties introduced by the possibly varying requirements in the matter of specimen preparation between prescreening and screening The variation is probably most marked when a machine is expected to work with the same preparation as a man. Man tends to prefer complex, many colored stains often with poor contrast and delicate colors, machines tend to prefer simple single stains and as much contrast as possible a crowded field is advantageous for man whereas machines prefer objects separately placed man is said to gather information from clumps of objects and can certainly recognize the constituent parts of a clump more easily than a machine man tends to prefer wider fields of view than machines, man uses irregular speeds a machine either a constant speed or regular steps man uses a single sensor and through that sensor works at various resolutions and with a variety of parameters they are all combined in the one set of eyes and brain, but a machine tends to become divided if it is made to operate at various resolutions and measure a number of parameters.

Track Transport Prescreening and Screening

The use of tracks has arisen from the desire to improve on conventional slides and filters. Though a nib or tube could be used to lay a track on a slide this has not been seriously entertained though attempts have been made to improve on repeatability and dispersion of objects using slides

Various track laying devices have been proposed for making tracks of cells suitable for presentation to a microscope these include two bacteria detectors using either plastic film or a glass tape or a disc a cervical cytology prescreening device a cell dispersing device and a device as part of a cervical smear analyser and reader project (CESAR)[3] The bacteria detectors use impactors to direct the objects onto the substrate. The prescreening device and the cell dispersing device use tubes to deposit the material and the CESAR device uses a trace laying nib.

The latter three track laying devices reflect different *approaches* and different estimations of the requirements for prescreening and screening The cervical cytology prescreening device copies the conventional slide preparation as far as possible using conventional fixatives and staining methods, staining with Papanicolaou stain after laying on the film The track laid from tube, is mostly over one millimeter in width and the edge is *irregular* The cell dispensing device allows drops of material stained in suspension by Feulgen reaction to fall onto film and spread freely The CESAR device uses material prestained in suspension with methylene blue and special nib to lay narrow tracks of constant width The tracks having regular edge can be of selected width between 1000 and 100 microns.

The problems of the slide which track preparation hopes to overcome include 1) uneven spread of material with resulting heaps and open spaces 2) uneven staining, in part due to heaping of objects one on top of another 3) the necessity for zigzag sensing over wide area 4) the lack of definite edge to the preparation being sensed, 5) the difficulty of location of and recall to particular objects in particular samples, 6) the difficulty of holding the objects to be sensed in the focal plane of the system, and 7) the difficulty of making several measurements on particular sample

The way in which the problems listed above may be tackled with track preparation are as follows. 1 and 2) Specimens collected into liquid can be treated by standard preparative schedule to obtain more even spreading than on slide The degree of dispersion attempted can be tempered by the consideration of the loss of information that may follow from too vigorous action 3 and 4) If the track is sufficiently narrow and has regular edge the whole width can be encompassed in single passing through sensing device or microscope field of view 5) Marking of the film by hole punching or making signals on magnetic stripe together with serial numbering of samples allows for the use of an automatic location and recall system The correspondence to particular object is easier with narrower tracks 6) The surface of the film carrying the track can register against datum on either side of the track The narrower the track the nearer the datum can come to the objects sensed 7) Successive measurements can be made on the same object as it travels through number of sensing stations

Summary

Prescreening has been discussed with reference to the nature of, cytology screening, man and machines Preparation of material for sensing in suspension and on solid substrates has been noted. Track transport has been considered in greater detail and comparison made between various tracks and between tracks and conventional slides.

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Dr. Tolles

Development of Standards

Routine Cytologic Screening

As starting point for this discussion, simplified, but formalized view of the function diagram of today routine cytologic screening procedure is shown in the upper part of Figure 1

At the left is the collection of the specimen It progresses to the right for processing and preparation for *inspection by the cytotechnologist* The latter often is asked to make only three decisions

(1) there is insufficient material to allow screening judgment (INS)

(2) specimen contains cells of such abnormal or suspicious characteristics that review and diagnosis by the pathologist is needed (S)

(3) no abnormal or suspicious cells, no further review (N)

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General Performance of Prescreening Systems

The last 15 years have seen a half-dozen[1–4] technical embodiments of the prescreening concept, each distinguished by one or more interesting and different technical or operational features. These include flow-through systems, cell-selector systems, and systems which measure cells deposited on slides or tapes. However, to date, none has achieved clinical maturity in technical performance or economic performance. Without being too detailed, a review of the reports of the clinical trials of a few of these systems is revealing, and will contribute to future design and evaluation.

Those systems which have undergone clinical trials report that of the specimens *which are accepted by the system*, the false negative rate was acceptably low and that the false positive rate was of the order of 40 to 60 per cent. On the face of it, such a false positive rate would reduce the work load, or increase the product of the technologist by a factor of two. Some believe that even this low multiplier would be a useful starting point from which to improve the system effectiveness, some do not.

In addition, one must consider the throughput factor in order to have a more complete basis of evaluation. Some of these systems[2] report a throughput factor of the order of 50 per cent, another[4] achieved a value of 85 per cent. Clinicians clearly understand the meaning of a low throughput factor. It means that only a fraction of the subjects presenting themselves for examination will receive it the first time. They will be asked to repeat the procedure and may, in fact, again fail to receive an examination. Aside from the inconvenience of recalling patients, one must be assured that nearly all the presenting subjects will be examined.

The Throughput Factor

In order to endow the throughput factor (k_p) with more than an intuitive value, it can be shown that the probability (P^-) of not receiving a prescreening examination after n exposures is

$$P^- = (1-k_p/100)^n \qquad (1)$$

where k_p is the throughput factor $0 \le k_p \le 100$, n is the number of exposures.

Table I shows the percentage of patients who will not receive a prescreening examination as a function of the throughput factor and the number of exposures which are required.

Table I *Percentage of Subjects who will not Receive Prescreening*

Number of Exposures (n)	Throughput Factor k_p				
	50	70	80	90	95
1	50	30	20	10	5
2	25	10	4	1	2
3	13	3	1	1	
4	7	1	2		
5	3	3			

Suppose that the throughput factor is as low as 70 per cent. The first time through the system, 30 per cent of the specimens (patients) will be rejected by the system, and must be asked to submit a second specimen. The second time 30 per cent of the originally rejected 30 per cent, or 10 per cent of the original subject group will fail to be examined. The third exposure will reduce the examined percentage to 3 per cent, and the fourth exposure will reduce it to 1 per cent. Clearly no diagnostic service can operate with this high a rate of recall.

If, on the other hand, the throughput factor is 90 per cent, we see that a single recall of 10 per cent of the original group will result in the failure of only 1 per cent to be prescreened.

Thus, the throughput factor emerges as a crucial parameter in the prescreening process, and one which will place a heavy burden on the instrument design and manufacturer.

Guidelines for Performance of Prescreening System

The false positive rate should certainly be lower than 50 per cent. A rate in the region of 25 per cent or less is a significant improvement in screening efficiency. The minimum that is likely to be attainable is in the neighborhood of 10 per cent.

The false-negative rate must be kept as low as possible. Many references in the literature suggest 5 to 10 per cent as acceptable, though it is obvious that the lower figure the better. (The false-negative rate and the false positive rate are not independent; they are usually linked in a hyperbolic relationship (FN × FP = const) so that improving one variable leads to degradation of the other.)

The throughput factor of the prescreening instrument must be at least 90 per cent in order that no more than 1 per cent of the input subjects escape the prescreening operation (one recall being permitted).

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1. Boddington, M. M., and Diamond, R. A. Apparatus for automatic preparation and scanning in cervical cytology. Brit. Med. J. 3: 160–161, 1967.

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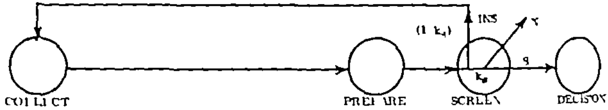
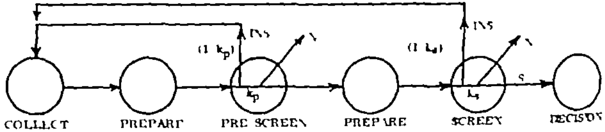


FLOW DIAGRAM FOR ROUTINE CYTOLOGIC SCREENING



FLOW DIAGRAM INCLUDING PRE SCREENING

FIG. 1

The following general levels of performance may be expected and of course vary from one demographic and clinical situation to the next

(1) About 3 per cent of the smears may be unacceptable for the purpose of decision-making This corresponds to a throughput factor (k_s) of 97 per cent

(2) The technician will bring from 1 to 5 per cent of the slides screened to the attention of the pathologist. Even though the incidence of cancer is well less than this, such referral does not represent false positives as much as the acknolwedgement of the presence of other pathologic entities which may require action.

(3) Thus, the technologist declares that 92 to 96 per cent of the specimens received are negative The percentage of cancer patients missed and called negative (FN) has been reported to be as high as 15 per cent, but it is generally felt that it is substantially less when good collection procedures are employed

The technologist is a true screening agent reducing the slides to be reviewed by the pathologist by a factor of ten to twenty five At the same time the concentration of so much decision making in one person creates a limitation of volume handling capacity

The Prescreening Concept

Pre-screening is a concept enunciated in the mid-fifties which visualized an instrument interposed between the specimen collection and the technologist The principle function of the pre-screening device is to deflect the unequivocal negative specimens from the need for human inspection It should do this with little or no compromise in the diagnostic capability of the system as a whole and, at the same time, perform this function at a reasonable cost.

The lower diagram of Figure 1 shows the addition of the pre-screening function to that of the routine scheme Some differences are apparent The first is that the specimen will have to be prepared in a manner required by the instrument (which may or may not be satisfactory for subsequent human review) *To* aid in this comparative analysis, let us require the prescreener to accomodate to the same decision triad as that of the screener in the last example A second difference from the routine model is seen in that the prescreener will introduce their own throughput fraction k_p and an INS fraction of (1-k_p) The INS fraction, if small, will not constitute more of a deficiency than in the routine case but clearly if it should rise to the order to 20 to 30 per cent the diagnostic performance of the system will be badly degraded as far as cancer detection is concerned, and cause an intolerable call-back ratio The throughput fraction k_p will divide its output into negative (N) decisions which will constitute the direct reduction of workload on the technician, and into suspicious (S) decisions which will be forwarded for human judgment.

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DRS. ROSENBERG AND KLINE MESSRS. LEDEEN AND MARKOWITZ

DISTINGUISHING NORMAL FROM MALIGNANT CELLS ON PAPANICOLAOU SMEARS USING COMPUTER MEASUREMENTS OF STANDARD MORPHOLOGIC CRITERIA

Accurate measurements of cell morphology are difficult to obtain While some workers have performed large numbers of measurements manually the time-consuming nature of this approach precludes its wide application Recent attempts to use the computer to help automate measurements of cells have met with some success and are discussed elsewhere in this symposium

In this symposium we would like to summarize our own results on the measurements of cells on Papanicolaou smears using a programmable visual-input computer system The use of decision theory to aid in the selection of the best criteria for distinguishing normal from malignant cells is also presented

Materials and Methods

Cell on routinely prepared and stained Papanicolaou smears of the uterine cervix were used for all measurements Normal and malignant cells as diagnosed by a cytopathologist were photographed at 100x magnification using Kodak Panatomic-X film.

A programmable film-reader (III PFR) interfaced to a high speed digital computer was used to make the measurements.

The light source of the film reader is a cathode ray tube emitting a sharp pencil of light at the x and y coordinates specified by the computer This light beam is split, one beam going to a reference photomultiplier and the other carefully focused to pass through a very small area on the photographic film. The amount of light passing through the film—a measure of the density of the film in that area—is measured by a second photomultiplier The signals from the two photomultipliers are then compared to determine the transmissivity of the small area on the film. This information is relayed to the computer and a new set of x-y coordinates to be measured is determined. This system is not suitable for direct measurement through the microscope A system we have designed for direct microscopic viewing will be described elsewhere.

Computer programs were developed to identify individual cells on Papanicolaou smears (avoiding overlapped cells, debris, dust, etc.) and to automatically measure the size, shape, and density of individual cell nuclei. The average radius of each cell nucleus was determined by measuring over 1024 radii of each cell in different directions from the center The average deviation of these radial measurements from the average radius provided a measure of nuclear irregularity Summing the areas of the triangles defined by adjacent radii resulted in a measurement of the nuclear area Th density of measured points within the nucleus were used to calculate the average nuclear density

Choosing a Threshold

Determining the threshold transmissivity value effective in separating cell nuclei from cytoplasm and background fields is an important first step in performing cell measurements. We therefore tested the effect of varying the threshold on the results of cell measurements. Figure 2 gives the results of measurements on the cell nuclear radius, irregularity and average density at a variety of arbitrarily chosen thresholds. As might be expected, the measurement of cell size (i e. radius) varies greatly with threshold. Measurements of cell shape and density were relatively independent of threshold, however For the measurements to be described a threshold value was selected which provided the best visual differentiation between the nucleus and cytoplasm and this same threshold used for all measurements

Selecting Cells

Computer programs were developed to identify individual cells in the microscopic field. Cells selected by the computer could be shown on video display for human examination In only rare instances was extraneous material mistakenly identified by the computer as an individual cell

Reliability of Computer Measurements

Computer and hand measurements of the average nuclear radius were independently performed on 12 normal cells. Hand measurements were performed on enlarged photographs of cells The correlation coefficient relating computer and hand measurements was 0 98

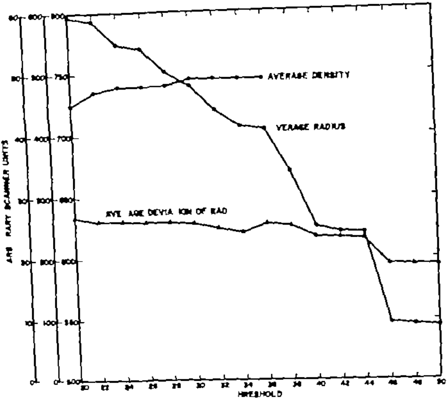

Fig 2 Effect of varying threshold on different cell measurements

Reagan used tedious hand techniques to measure the nuclear radius of cells and obtained an average radius of 3.37 ± 1.2μ for normal cells[1] on Papanicolaou smears. This is very close to our computer obtained average of 3.38 ± 0.66 μ.

Measurements of nuclear radius, irregularity, area and average density were repeatable to within 1 per cent.

Morphologic Parameters of Normal and Malignant Cells

Normal and malignant cells as identified by pathologists were measured using the computer system. The results of these measurements are given in Table II and Figures 3 and 4.

It is readily apparent that significant measurable differences exist between normal and malignant populations of cells. However, significant overlap between the two populations also exists. For any single morphologic parameter, cells in the region of this overlap cannot be graded as either normal or malignant. It is thus apparent that it will be necessary to use measurements on many different parameters to distinguish normal cells from malignant ones.

TABLE II *Computer Measurement of Normal and Malignant Cells on Papanicolaou Smears*

	Normal	Malignant*
Radius (μ)	3.38 ± .66	5.43 ± 2.18
Area (μ²)	39.11 ± 14.77	119.08 ± 92.97
Irregularity	60.84 ± 22.12	127.37 ± 49.94
Density	39.5 ± 3.9	42.8 ± 7.5

* Invasive squamous carcinoma cells

Data Analysis

There are two sides to the problem of evaluating measurements of cells. The goal, of course, is to identify malignant cell as malignant. It is bad if normal cell is misclassified as malignant. It is also bad (though not necessarily equally bad) if malignant cell is mis-classified as normal. These types of errors must be balanced against one another so as to produce the best decision for any given cell. As shall see the question of best while not immediately obvious is quantifiable and that in sense, is the point of this section.

Let us make the simplifying assumption that

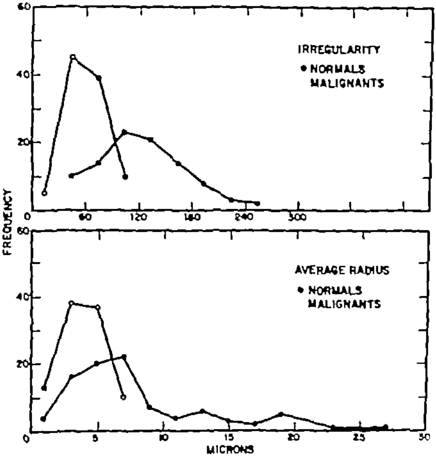

Fig 3. Computer measurements of the morphologic parameters of radius (top) and irregularity (bottom) of a large number of normal and malignant cells.

there are two possible alternatives—a cell is either malignant or normal Inasmuch as we are going to classify a cell as malignant or normal there are then four possible outcomes of each decision These are shown in Table III In order to be able to evaluate any set of cell measurements or any decision scheme based on these we need to know the relative frequencies of the various outcomes. We need concern ourselves only with two (independent) outcomes, say outcome 1 and outcome 2. (Knowing the relative frequencies of these two, we can calculate the relative frequencies of each of the others.)

The relative frequency of outcome 1 is an estimate of the conditional probability of calling a cell malignant given that it is malignant denoted

p (MALIGNANT | malignant) or P (M | m)

Similarly the relative frequency of outcome 2, an estimate of the probability of calling a cell malignant which is in fact normal, is denoted

p (*MALIGNANT* | normal) or P (M | n)

It turns out, both theoretically and practically that the two conditional probabilities are related to each other and that it is their relation that is of interest. That is we can increase the probability of outcome 1 by changing our decision criterion but with this will also increase the probability of outcome 2 For example consider Figure 3 (bottom) which shows a normal and a malignant population of cells distributed along the dimension of average radius

Suppose we adopted the simple criterion of calling a cell *MALIGNANT* if its average radius is larger than say 6 0 μ Then we find that p (M | m) = 38 and p (M | n) = 11 If however we changed our decision criteria to, say 4 5 μ we find p (M | m) = 74 and p (M | n) = 39 Finally if we were to use 3 0 μ as our decision criterion then p (M | m) = 87 and p (M | n) = 77

Notice that by changing the criteria we have changed the relative frequencies of the various outcomes but we have not really improved our classification To understand this, let us plot *p* (M | m) against p (M | n) as in Figure 5

We have drawn a smooth curve through the points (including the origin) Any point on the curve can be achieved by picking the appropriate criterion. Exactly which criterion we choose depends on how costly missing a malignant cell *is* compared to falsely classifying a normal cell as malignant However we cannot do *better* in the sense of being above the curve no matter what criterion we use as long as we confine ourselves to

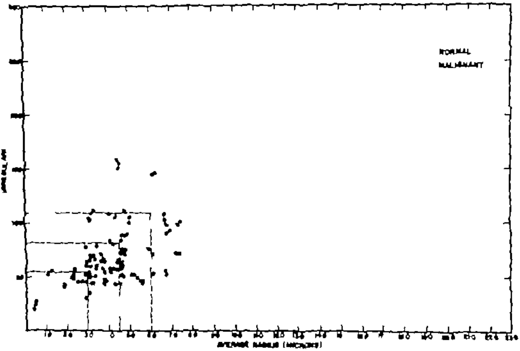

Fig 4 Distribution of the average radius and irregularity of normal and malignant cells

TABLE III *The Four Possible Outcomes of the Cell Classification Problem*

WE CALL THE CELL	A CELL IS EITHER Malignant	 Normal
[illegible]	OUTCOME 1 GOOD	OUTCOME 2 BAD
[illegible]	OUTCOME 3 VERY BAD	OUTCOME 4 GOOD

erage radius measurements Now let us, in fact, consider another dimension—that of irregularity

Reference to Figure 3 (bottom) shows that if ecall all cells ith an irregularity measurement greater than 110 MALIGNANT then p (M | m) = 58 and (pM |) = 042 If change our criterion to an irregularity measurement of 80 then p (M | m) = 78 and p (M |) = 19 Finally for criterion of 35 p (M | m) = 64 Plotting these values in Figure 5 yields curv every here abov the average radius curve. Thus, irregularity provides the better discriminator of the two In fact, the higher the curve the better the criterion used to generate it This is the point of using this type of analysis to evaluat criteria for separating normal from malignant cells

Though in the above *description we have used* only single decision criterion, any umber may of course be combined in deciding between normal and malignant cells and the bove method used to evaluate the benefit derived from this combination Let us take the obvious next step of combining the two dimensions, average radius and irregularity For convenience we have taken the same criteria along each dimension using Figure 4 and made our decisions based on both criteria The results are shown in Figure 5. As you can see there is small improvement *1 using the two criteria* jointly That is the combined curve is higher than that based on only single kind of measurement The improvement would be greater if the average radius and the irregularity of each nucleus were totally independent of each other

T emphasize the important point of this section evaluating any set of measurements or decision criteria based on them, t is necessary to consider both the probability of misclassifying normal cells as malignant and malignant cells as normal. Only hen both probabilities are know can the efficiency of two sets of measurement be compared

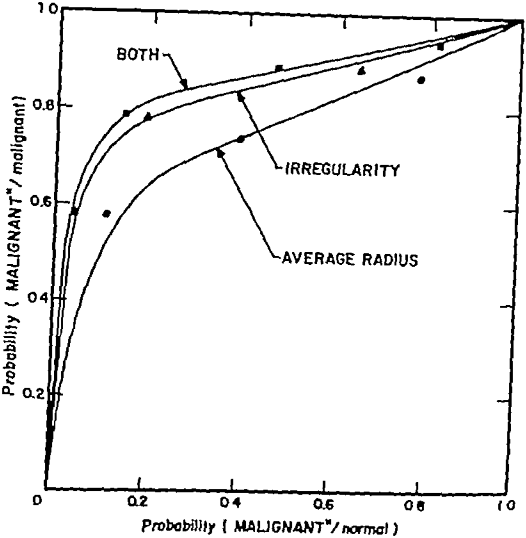

Fig 5 Evaluation of criteria for deciding between normal and malignant cells.

Conclusion

We have presented our studies using a computer automated visual input system to distinguish between normal and malignant cells on Papanicolaou smears. Accurate and repeatable measurements of the morphologic parameters of individual cells can be made and significant differences found between malignant and normal cell populations. Deciding whether *any individual cell is normal or malignant* is far more difficult, however and requires many kinds of measurements on any *single* cell. An application of decision theory to provide an objective evaluation of criteria used to separate normal from malignant cells *has* been presented

Current work in our laboratory is proceeding on two main fronts. A computer system based on the use of the *image* dissector tube capable of analyzing cells directly through the microscope has been built and is currently being evaluated. We are also continuing to evaluate *new* measureable cell parameters that may be of use in distinguishing normal from malignant cells.

REFERENCES

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DR. DIAMOND

SPECIAL CONSIDERATION OF SINGLE PARAMETERS

Unsuccessful attempts have been made over many years to devise a machine capable of automatically sorting cervical smears into two categories one to be rejected as negative and the other to be examined by the human eye

Ideally one should construct a machine which is capable of studying the cells rapidly and picking out those which vary from the normal, *i.e. it should* be capable of doing the same work as a trained human observer However the human eye is very sensitive and complex pattern recognition system and it will be many years before computer eye is able to compete with it on this basis

In view of this it was suggested by Mr F H. Smith, of Vickers Instruments[1] that if a feature could be found which is always present in positive smears, it would be sufficient if this single parameter could always be detected. The obvious feature to use would appear to be nuclear size

Instead of studying the whole range of nuclear diameters special investigation was made of the occurrence of unusually large nuclei, In the right hand tail of the nuclear—diameter distribution. In the initial series[2] it was found that, of 100 "positive smears from cases with carcinoma-in-situ and dysplasia, all had at least 12 nuclei with shortest diameter of 12 μ, while the majority had many such cells (up to 660)

The hypothesis that nuclei greater than 12 μ in their smallest diameter were always present in positive smears and infrequently present in negative smears was independently assessed by Rimmer[4] although her results were basically in agreement, she stated "that no conclusion may be drawn until much larger sample is examined

A further assessment of this hypothesis was made using larger series In this series conducted at the Institute of Clinical Pathology and Medical Research in Sydney 256 positive smears were examined and all contained large nuclei (i e nuclei 12 μ in their smallest diameter) In 217 (85%) large nuclei were found on the first line of the slide that was scanned. In 36 (14%) large nuclei were less readily found, but complete scanning of the smear was unnecessary In 3 cases complete scan of the smears had to be made In the same series 1000 routine negative smears were examined and of these 730 (73%) contained no large nuclei, while 260 (26%) had large nuclei It was noted that large proportion of those negative smears containing large nuclei were post menopausal and post-natal smears. This series would appear to substantiate the hypothesis Thus, if device could be perfected which would mark every field containing nucleus of 12 μ diameter or more it is unlikely that any positive smear would be missed This machine would also only draw attention to 25 per cent of negative smears

Using the Vickers Cytology Screening Apparatus[1] cervical cells can be spread and stained on continuous tape and attempts are now being made to incorporate prescreening device to detect large nuclei and mark the tape at those points However the main problem with this device is the rejection of various artefacts such as rolled or overlapping cytoplasm and pieces of dirt and grit It should also be mentioned that this device relies on nuclear density to enable it to measure nuclear size.

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DRS SHORTER AND PRIBOR MR. THETFORD
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Conventional screening of Papanicolaou stained smears by any cytologist takes advantage of a vast store of information accumulated, organized and correlated within his brain The combined hand, eye and brain of this trained individual obviously operates in most complex multidirectional parallel processing manner to rapidly and accurately interpret the visual patterns presented in the specimen The slide under evaluation is matched with patterns stored in his memory and almost instantaneous correlation of various parameters of given cell with descriptors of known normal and abnormal cells and their characteristics takes place. All this occurs on the order of milliseconds The screener also compares not only individual cell characteristics but the relationship of cells to one another Many individual items are taken into account as he reads the slides and these factors are all well known To name a few these include cell size, nuclear size and nuclear/cytoplasmic ratio, staining characteristics, nucleoli, chromatin distribution, cell clustering, dirty background" and many more He quickly determines the presence of artefacts which may affect the interpretation, diseases and altered physiologic states, other than premalignant and malignant disease are also readily detected Clinical information, of course, is also taken into account in his reading of the slide.

With this brief background, it is easy to appreciate the difficulties to be encountered in attempting to design an instrument capable of adequately screening or prescreening cytological material The approach to development of instruments to analyze cells can be made at two levels one to

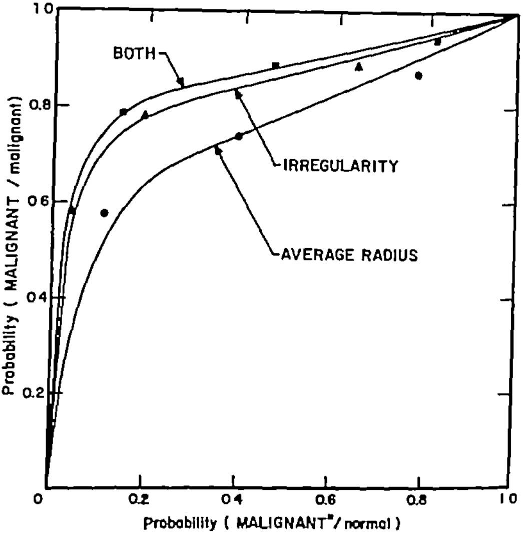

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Image processing technics have been disappointing to date and require complex and expensive equipment.[9] Even with computers of microsecond data acquisition capability that are coupled to scanning instruments able to quickly and accurately traverse a cell image, the many data points necessary to evaluate characteristics of even a single cell still take a considerable amount of time. When we add to this the fact that many thousands of cells must be analyzed in the usual screening, we can begin to understand the magnitude of this problem.

One of the easiest properties that can be measured by machine is the transmission or absorption of light in the visible or ultraviolet range. By controlled scanning, a matrix of light transmission values, for example, can be obtained from which cell characteristics can be determined such as cell size and nuclear/cytoplasmic ratios. The TICAS project of Wied, *et al.* which measures extinction values of ultraviolet light and performs some sophisticated analyses on this data is a good example of the extent to which a single parameter approach can be carried. However a significant drawback to the light transmission studies is the requirement for carefully preselecting the cells to be analyzed in order to obviate artefacts such as overlapping and cellular debris.

The CLSAR system of Dawson, *et al.* while not fully operational, has a unique method of deposition of cells on a moving strip in which many of the overlapping artefacts are eliminated when the cells are placed on the strips in an essentially unicellular layer.

Light dispersion, absorption or transmission may also be performed on cells flowing past a sensing device. Crucial focusing of the light beam, narrow channels to permit passage of cells in single file and problems with clogging must be overcome. To avoid sampling errors, it would be useful to be able to recover the cells that have flowed through the analyzer for reruns or for visual inspection after staining. Specimens may be collected by a wash technic or by swab scrape method following which the collected cells are suspended in a preservative solution. An example of this approach has been described by Koenig *et al.*

A novel method of determining the amount of nucleic acid in cells (on average) has been described by Roth. It requires the counting of cells, addition of an acridine dye which in proper concentration binds quantitatively to the nucleic acids, and subsequent measurement of residual dye remaining in solution or measurement of total emitted fluorescence. This approach appears relatively simple to automate. Well-differentiated tumors may not have a significantly increased or altered amount of nucleic acid and the number of cells with abnormal amounts of nucleic acid must be relatively high compared to the normal cells to detect differences. Advantages to this approach are the massive numbers of cells which are subjected to analysis at one time, the fact that the cells may be reharvested for visual reading and the relative ease of performing the test run.

In 1964 Peckham and Ladinsky[3] showed that normal cells from the vaginal pool have a small volume despite their large two-dimensional appearance on the slides. Further they demonstrated that in cases of carcinoma of the cervix and endometrium, a second population of cells was present and that these cells had a five or sixfold increase in average cell volume compared to normal cells—despite the fact that they usually often appeared to be smaller. They used a Coulter counter to determine the relative numbers of cells with varying cell volumes and in each case showed a peculiar curve type with a noticeable second population peak.

At the Institute of Laboratory Medicine, Pribor *et al.* are presently working on a modification of this technic which couples the conduction cell apparatus with a pulse-height analyzer and computer "on-line" for the storage and analysis of the data. They have screened over four thousand specimens obtained by vaginal irrigation and have compared conventional Papanicolaou smears taken at the same time with smears and cell volume profile curves made from the irrigation specimen. Four distinct types of curves were noted. Correlation with tissue diagnosis indicated that almost all of the dysplasias, carcinomas *in-situ*, and invasive carcinomas were associated with two of the curve patterns. There was a high rate of "false-positive" curves which approximated 45 per cent. In addition, there were about 10 per cent of specimens which could not be run on the automated instrument because of very low cellular content.

Several investigators including Odell and Peckham[8] have been using metabolic activity of exfoliated cells and of vaginal pool material in an attempt to distinguish normal from abnormal cases. Measurements of various enzymes including β-glucuronidase, α-mannosidase and 6-phosphogluconate dehydrogenase have been demonstrated to show differences in abnormal cases. The incorporation of amino acids tagged with radioactive nuclides has also been shown to be different in normal and abnormal cells. Amino acid incorpo-

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choose single or multiple parameters of sufficient sensitivity in order to detect changes abnormal enough to flag the attention of the cytologist who then visually reads the specimen or two, to develop an instrument capable of interpreting and actually diagnosing the cellular changes based on rigidly defined criteria such as those utilized by the *cytologist.*

Prescreening instruments appear to be *simpler* to construct. Many cellular characteristics can be measured by a variety of instruments. The prescreening of material implies that an instrument *will detect cell changes and bring these to the* attention of the screener who would *then proceed* to diagnose the case. Ideally the instrument would miss no positive cases even though we would accept the situation whereby the instrument presents many *false* positive cases for final visual interpretation. Of course, we must be able to adjust the sensitivity of the instrument to accomplish this On the basis of some past studies, it is estimated that only 85 per cent of known or *proven cancers are associated with a "positive"* cytologic smear on a random single specimen basis.[4, 8, 11] Instrument analysis should be made to match this at least On the other hand, the reason for any prescreening technic is basically *the elimination of the bulk of tiresome work per*formed by the cytology technicians in order to reduce significantly those 993 negative cases he sees in finding the approximately seven positive cases in a thousand. Our own feeling is that the *instrument approach should reduce by well over* a half the number of cases to be manually screened

One obvious deficiency of any prescreening instrument is the loss of some clinically useful information other than detection of cellular ab*normalities* These include the estimation of hormonal effect and alterations of the microbiological milieu. For the most part, development of instruments has proceeded along the line of detecting only *normal and abnormal or malignant* cells.

The analytical method to be employed and the parameter to be measured have an important bearing on the type of specimen that must be collected *In general,* there are three types of specimens that can be utilized

1. Static cells affixed to a slide or support medium—these cells may be preserved or living
2. *Cells in a* liquid suspension—these cells may also be preserved or live.
3. Living metabolically active cells in *liquid* or on solid growth media, or *their metabolites*

The third *method does not* appear to be suitable *for mass* screening at this time and collection, transportation and processing *would be a formid*able undertaking in any but a research endeavor

Obviously any system using cells in the living state has a strict limitation in regard to collection, preservation and processing Cells collected and *preserved in a fluid medium can be handled in a* variety of ways and conventionally stained slides can usually be made from the specimen with excellent agreement with the Papanicolaou method [1, 2] Collection methods must usually be *rigidly controlled for best results with any instru*ment technic in order to reduce or eliminate the effect of artefact that might otherwise be introduced

Many investigators have sought to develop *automated methods for detecting cellular ab*normalities. Some parameters that have been evaluated with the type of specimen required are listed below

1. Static cells fixed on slide—image processing *by eye and machine*
   a. Cell size
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   c. Staining characteristics, density color
   d. Quantitation of RNA and DNA RNA/DNA *ratio*
   e. Fluorescence (antigen/antibody)
   f. Transmission and absorption of light and UV light
   g. *Shape of nucleus*
   h. Nucleoli
   i. Nuclear/cytoplasmic ratio (based on area)
   j. Nuclear chromatin content
   k. *Nuclear chromatin pattern*
   l. Eccentricity ratios of nucleus
   m. Degree of cellular variability pleomorphism
   n. *Pattern recognition*
2. Flowing cells or cells in suspension
   a. Cell volume profiles
   b. Membrane impedance
   c. Differential sedimentation
   d. *Light* and absorption and/or transmission
   e. Specific gravity
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   h. *Dye absorption*
3. Living cells
   a. Metabolic activity

Since the greatest body of knowledge concerning cancer detection *by cytological means* is based on visual criteria, it is not surprising that many mechanized approaches to concern screening also use the visible properties of cells as their basis The primary problem *in automating the screening* process using a single or very few measurements is the definition of the appropriate acceptable criteria This is well described by Tolles *et al* While the cytopathologist *can mentally process* many informational items simultaneously the machine examines one item at time and there must be rigorous definition of each parameter measured in order to obtain consistent and satisfactory result

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In 1964 Peckham and Ladinsky[illegible] showed that normal cells from the vaginal pool have a small volume despite their large two-dimensional appearance on the slides Further they demonstrated that in cases of carcinoma of the cervix and endometrium, a second population of cells was present and that these cells had five- or sixfold increase i averag cell volume compared to normal cells—despite the fact that they visually often appeared to be smaller They used Coulter counter to determine the relative numbers of cells with varying cell volumes and in each case showed peculiar curve type with a noticeable second population peak

At the Institute of Laboratory Medicine, Pribor *et al* are presently working on modification of this technic which couples the conduction cell apparatus with pulse-heigh analyzer and computer on-line for the storag and analyses of the data They have screened over four thousand specimens obtained by vaginal irrigation and hav compared conventional Papanicolaou smears *taken at the same time with smears and cell* volume profile curves made from the irrigation specimen Four distinct types of curves were noted Correlation with tissue diagnosis indicated that almost all of the dysplasias, carcinomas in situ, and invasive carcinomas were associated with two of the curve patterns There was high rate of "false-positive curves which approximated 45 per cent In addition, there were bout 10 per cent of specimens which could not be run on the automated instrument because of very low cellular content

Several investigators including Odell and Peckham[illegible] have been using metabolic activity of exfoliated cells and of vaginal pool material in an attempt to distinguish normal from abnormal cases Measurements of various enzymes including *β*-glucuronidase α-mannosidase and 6-phosphogluconate dehydrogenase have been demonstrated to show differences in abnormal cases. The incorporation of amino acids tagged with radioactive nuclides has also been show to be different in normal and abnormal cells Amino acid incorpo-

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ration is very high in material contaminated with trichomonas and monilia and presumably these infections would have to be eliminated before under taking this testing procedure

To date, to our knowledge no single parameter measurement method has effectively solved the problem of automating a prescreening or screening method for exfoliative cytology Single parameter approaches have obvious difficulties associated with most of them and possibly the cascading of two or more single parameters will increase the sensitivity and accuracy of detecting malignant cells within large numbers of normal cells

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DR. HUSAIN

Special Consideration of Multiple Parameters

The title "Approaches to Prescreening" permits wide interpretation. It demands primarily optimum *cell sampling* and *cell presentation* techniques to submit the malignant cell in its best form for identification. Both these procedures are dealt with elsewhere in this Symposium and the mode of their achievement will not be considered here in detail. The quality and character of the final presentation will, however dictate what parameters are recognizable by automatic scanners and must therefore be defined

Cell Sampling

Bearing in mind that the instrument is designed to scan cell populations where the abnormal or malignant cell rate can vary from very few (and certainly less than 500) to over 1245 cells per slide (11) (15) and that the ratio of abnormal to normal cells can be 2 5/100 in the vaginal sample, 35/100 in the cervical sample and 110/100 by endocervical cotton-type applicator[11] the choice of sample would seem obvious. There are, however reservations in that cell clusters are unreadable by automatic scanners except by fairly slow scans at high magnification, and the "buried signals" (i.e. cells in unreadable clusters) in a series of squamous cancer case smears analyzed by the author from scrape smear samples was found to be 10 1 This brings such forms of collection as the irrigation smear with its more evenly distributed and nonclustered cell sample in for serious consideration

Cell Presentation

Cluster disruption and cell dispersal techniques The use of enzymes such as trypsin or papain, or by hyaluronidase or cell dispersants such as versine urea or various polymers and even ultrasonics, must leave the characteristics of nucleus and cytoplasm intact otherwise the disruption of such cell relationships may destroy those very parameters utilizing the more stochastic processes of known transition probabilities such as the association of cytoplasm surrounding a nucleus, or the marginal positioning of a glandular cancer nucleus, even the patterns we recognize in the cancer diathesis" At the higher degrees of resolution the finer descriptors within the nucleus would be lost if depolymerization occurred to any extent through processing

Also it is obvious that a simple fluid suspension of cells, rapidly scanned at different wavelengths and scored by photomultipliers for the ultraviolet light absorption maximum of DNA against the visible light absorption content giving nuclear/cytoplasmic ratio such as with Kamentsky's apparatus[8] will have much greater limitations on the use of finer nuclear discriminators than cells fixed and stained and spread out on slides or transparent tape or film strip.

Single versus Multiple Parameters

Again, balance has to be struck between the wide range of parameters available at high optical resolution (such as ×500 to ×1000) with the accompanying uneconomic slowing down of scan compared with the more limited approach using the few parameters identifiable at much lower magnification (at ×200 to ×250) and at considerably increased speed This latter problem provides the main theme for this article

Degree of Automation

Finally and perhaps the most important decision of all should the intention be to produce complete automation to diagnose malignancy from a cell sample or should it merely be to mark the malignant cell for final reference by eye This latter procedure then limits automation to prescreening and removes in part the criticism of those who believe cancer is a serious diagnosis to be seen and made by eye Thus, then demands that the cell presentation which includes the stain technique be recognizable by the cytologist

If this be the proviso then marking the cell is an important feature for recall and reference, either on the slide by grid reference system, or on film strip, either by punch holes (as in the Vickers' machine)[17] or by electromagnetic tape tracks (as envisaged by Tetronics Ltd) Lastly as variant of partial automation, should this only serve to crudely separate out malignant cells for more discriminative scans by machine or eye providing sort of cell separator

Perhaps before discussing the present situation word or two about the history of this problem would be instructive

Some of the earliest work carried out in this field was by Mellors, Glassman, Papanicolaou and Silver[8] Tolles, Horvath, Bostrom and Spencer[16] in the late 50' and early 60' presented series of interesting and valuable cell population distribution graphs plotting nuclear extinction coefficients (ΣN) or optical density against nuclear diameter in the various Papanicolaou classes of smears, and also the nucleus ΣN to the ratio of cell diameter to nuclear diameter The pattern emerging from their charts indicated sufficient difference to encourage the use of these criteria of nuclear diameter and density to be used in their developing instrument "The Cyto-Analyzer" This apparatus, developed by Airbourne Instrument Company[13,14] unfortunately failed to succeed mainly on three counts.

(i) it was set too ambitious a target—the intention to separate the malignant from the non-malignant cell samples.

(ii) Inferior electronics (those extant at the time) Considerable advances have occurred in vidicon and plumbicon tubes of recent date

(iii) Confusion over neutrophils producing pseudo-positive signals—the most potent cause of such an error

Spriggs and *Diamond*[14] set out to demonstrat that all, or at least the large majority of malignant case cell samples from the cervix contained nuclei over a certain size, be they (the nuclei) malignant or benign They selected 12 and 16 µ as useful threshold diameters The latter size presented fewer false positive signals but also possessed dangerously few true positive signals in certain cases The Vickers' slit (now spot) scanning machine was produced on the basis of this simple parameter analysis

Husain and his colleagues[7] further developed this thesis using the Quantimet Image Analysing Computer to demonstrate that density of the DNA stain (i the extinction coefficient) of the nucleus (ΣN) played an essential part in this detection as the malignant nucleus contained a greater concentration of DNA (apart from the amount) and therefore stained more intensely overall than did the benign nucleus They went further to show that stratification of signals occurred in the density of stain up-take by the nucleus (extinction coefficient Σ N) The malignant nucleus possessed the highest ΣN the dyskaryotic or dysplastic nucleus lesser ΣN but still higher generally than the hyperchromatic and hyperplastic (inflammatory) benign nucleus Moreover there appeared to be tangential line of division of ΣN between the malignant and non-malignant the larger the nucleus the lower the ΣN threshold could be set This is valuable at both ends of the scale the larger softer glandular or invasive squamous nucleus can still be separable and again the smaller more pyknotic malignant nucleus can be selected out only at the highest ΣN so that the lower limit of size can approach those non-malignant nuclei that are equally darkly stained These are the superficial squamous at 6 µ and the single neutrophils polymorphs at 8 µ Thus to read from 10 µ and upwards on a tangential threshold of ΣN would gather considerable number of true signals The exclusion of false positive signals by certain cancelling or chopper[17] criteria will be considered later

Further work on more detailed analysis is being pursued by Ward and McMaster [18] Wied, Bahr and Bartels,[1,2,38] Dawson, Heanley Heber Percy and Tylko[4] to mention but a few groups with published results

These workers have been examining the whole gambit of nuclear and cytoplasmic characteristics which relate to malignant change Both Wied and Ward s group are amassing this data on computer tape to seek useful descriptors, from the specific topographical relationships of extinction values in the cell image Both Wied's and Dawson s group have been creating in effect new classifications, the former by TICAS (Taxonomic Intracellular Analytic System)[38] and the latter by their "cybernetical cell classification"[4] using equiprobable range contours as templates. Wied and his colleagues have been testing membership of unknown cells or creating transition probability profiles composing histograms to demonstrate "clustering" of signals from related cells.

From this historical introduction we should now consider the many traditional criteria of malignancy that could be used as templates for the automated scanner to recognize malignant nuclei A short list of these is as follows

1 *Size of Nucleus* Both larger and smaller than normal sizes exist in malignancy though there is a useful phase in development of advanced dysplasia and carcinoma in-situ where there is a preponderance of enlarged dyskaryotic nuclei
2 *Shape of Nucleus* Simple and complex This creates a form factor and is shown by Reagan et[12] and others' work to provide pleomorphism to a greater degree in malignancy than in benign conditions This reaches its maximum in the invasive stage However it is a criterion of statistical value rather than one of identification of individual malignant nucleus in the pre-malignant and early malignant phase.
3 *Irregularity of Nuclear Margin* It is said that every malignant nucleus has an irregular margin. This is only true if very fine irregularities are included and these would be difficult to detect in high-speed, low-magnification scans. The presence of irregularity would help in supporting the identity of a malignant nucleus but occurs as much from degeneration as from dysplasia or neoplasia
4 *Density of Stained Nucleus* (overall extinction coefficient, ΣN) This has already been considered above
5 *Condensation of Nucleoplasm*

   a) within the body of the nucleus, including the nucleoli

   b) at the nuclear margin

   This chromatin condensation or the spatial distribution of chromatin certainly in the stained nucleus, provides the bulk of the malignant cell discriminators as we see them by eye It is the thick strands or clumps of chromatin with their intervening clear areas and their general spatial relationships that the cytologist considers when identifying malignancy It will leave the discussants to present their views on the less obvious and more mathematical measurements of a cell (both nuclear and cytoplasmic) leading to the evaluation of moments of inertia and other similar parameters characterizing malignancy Like most biological criteria these changes may often be missing, as in the soft spongy texture to the nucleus seen in some glandular and advanced squamous cancer or almost complete condensation to a small dark nucleus, usually a sign of nuclear death. The overall size of the nucleus, however still remains well above the normal or inflammatory nucleus undergoing pyknosis.
6 *Cell Size and Shape* Both these refer to cytoplasm where the maximum size increase is at the dysplastic stage and maximum anisocytosis or variation in shape and variation in size occurs with the invasive cancer
7 *Nucleocytoplasmic Ratio*

   a) This is one of the most classic criteria of malignant cell diagnosis The fact that the nucleus occupies more than one-third of the plane area of the cell does not of itself spell malignancy as many end-on glandular and stromal cells, some inflammatory deep squames and even histiocytes, polymorphs, and lymphocytes would so classify When linked to size and absorption coefficient (ΣN) of the nucleus it provides a useful factor

   b) A variant of the nuclear cytoplasmic ratio is the step density gradient of a nucleus with its surrounding cytoplasm to distinguish it from an artefact which usually has an abrupt edge Some loss of signals from stripped malignant nuclei would be the price of this feature though there are ways of overcoming this also
8 *Various ill-defined Nuclear and Cytoplasmic Discriminators* and all patterns such as the characteristic clustering of the adenocarcinoma cell, the disaggregation of the dysplastic cell pattern the cohesiveness and uniformity of the carcinoma-in-situ cell and the pleomorphism of the invasive cancer picture
9 *The Use of Special Stains* both nuclear and cytoplasmic The use of fluorescent stains and ultra-violet light absorption patterns.

The Choice of These Parameters

Perhaps we can dispatch the last item (9) quickly by saying that if a suitable stain or stains can be devised for malignant DNA or RNA with such high degrees of differentiation from the norm that unequivocal Yes/N signals result, then very little further needs to be done. The failure of the use of berberine sulphate and acridine orange ith fluorescence and feulgen and gallocyanin and other pure DNA stains in the white light range should not deter further search, and this is going on If this could be successful then a fluid transport system might well be the quickest and most convenient. I is the absence so far of these techniques which leads us to consider the other characteristics listed above. This is not to say that some form of rough pre-selection and separation for more discriminative scanning could not be effected using these DNA and RNA stains and one should keep this possibility in mind.

Both monochromatic and polychromatic stains should be considered Papanicolaou' stain is an example of the latter but is too complex to utilise effectively to provide an adequat signal to-noise ratio Experiments using the Quantimet have show that monochromatic scanning of poly-chromatic-stained sample with the monochromator set to scan at the absorption maximum of DNA, which should be well away from that of cytoplasm, ill increase the signal-to-noise ratio that is to say the malignant cell signal is high bove the cytoplasm/debris background signals A simple exercise with the Papanicolaou stain was performed Using a green filter which gives a high nuclear signal, the reduction of the eosin stain content reduced the noise value of the cytoplasmic component considerably

Consideration of criteria 1 to 7 above is influenced by one major break-line in automated scanning, and this must be discussed first

The Problem of Optical Resolution and Focusing I possible to scan t ×200 to ×250 and achieve picture points of around 1 μ, perhaps a little less (l resol particles and optical density to that degree of size) At that level uclear size, crude form factors, density and relatively crude peri nuclear irregularities and intranuclear condensation foci and the larger ucleoli may be resolved

Similarly nucleo-cytoplasmic relationships can also be recognized on crude basis Focusing may be dispensed with, or be of fairly coarse nature

The finer degrees of extinction coefficient discrimination in the nucleus however are unattainable until one reaches ×500 to ×1000 magnification, l achieves picture point resolution of 0 5 to 0 25 μ At these levels, focusing is essential and needs to be critical Also at this magnification the speed of scan is greatly reduced These discriminators, however are undoubtedly the ones which will be nearest to being specific for malignancy

The use of cytoplasmic measurements should be of more value than they are, as show from Reagan ork[18] on cell areas in square μ normal = 1604, dysplasia = 1087 carcinoma-in-situ 352 nd, invasive cancer 229 square μ th very reverse of what happens i the nucleus.

The problems of registering the bsorption coefficients of cytoplasm, which are not much raised in malignancy are probably the reason why over all size is not used more In fluid scans it may well be of greatest value, but here again largely in connection with nuclear/cytoplasmic ratio

The Use of "Chopper" Criteria Perhaps more important than the parameters of malignancy are the criteria for exclusion of the false positive signal. I effect, this will be as much the secret of success as the selection of parameters

Simple criteria such as over size (a dense particle over 25 or 30 microns which is obviously not one cell but probably cluster) must be cancelled Also the large or small abrupt-edged, completely opaque particle is bound to be an artefact and that signal must be quenched There are numerous intricate ways of achieving such exclusion

The Economics of Speed

To be economic the speed of scan by the human eye should be equalled, if not bettered, and thus the reading of 30 to 50 000 cells in approximately 3 minutes should be the target It is my contention that such speeds cannot be achieved at magnifications of over ×200 to ×250 unless multiple scanners in parallel are used, and that for the purpose of fast scanning only the simpler parameters of size, crude form factors and overall density can be used However a secondary scanner at higher magnification, doing either more detailed line or boundary scans, could analyse those nuclei so selected, thus bringing in the remaining parameters Slow detailed scanning then would only occur on the suspicious signals brought up by the fast scan sensor( )

The fast, low magnification sensor could then utilise certain 'chopper criteria to eliminate false positive signals which might, at higher magnification, be just the criteria characteristic of malignancy I would instance the use of picture point differences on consecutive line scans where the irregular outline of neutrophil nucleus producing edge-variation of within 2 or 3 picture points would spell out neutrophil or artefact and chop or cancel false positive signal The fine irregularities of malignant cell margin would not register as such except at higher degree of magnification It is in this way we come to the eventual choice of parameters to program the sensor( )

I leave the final choice open, as there are many variations of thi approach The discussants will doubtless wish to elaborate on their particular theme and out of this Symposium should emerge number of working hypotheses

I should like to conclude with one submission

Dealing with raw biological material for scanning it would be more prudent at this moment in time to aim for the identification of signals than separation of cell samples into malignant and non-malignant. The overlap is too great. However many parameters can be used in the pre-screening templates. Success is more likely and more confidently achieved if the final arbiter is the human eye viewing the signal

As experience is gained in parameter selection and scanning techniques, then the ultimate separation will be welcomed as a bonus.

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DR. WATTENBURG

System Design Approaches for Automated Cytology Prescreening

It is the author's opinion that any automated
or semiautomated cytology prescreening system
designed today which relies primarily on the
measurement of a single parameter such as cell
volume DNA RNA mass, etc as these parameters are now being measured will have great
difficulty competing with trained cytotechnologists in a *production* screening environment. Fur
thermore the primary requirement of no "misse
positives" and the practical requirements o
processing rate and efficiency of selection (e
richment of final specimen for manual screenin
raise grave doubts as to the ability of a sing
cycle system meeting all of these requiremen
Consequently it is proposed that any design a
implementation effort toward achieving a pr
tical automated system give serious considerat
to the use of a multiple parameter multi-cy
system as described herein

The Problem

Wied has described the problem in its esse
form

1 The prescreening system must not mis
present tumor cells, i e no "missed positi
2 The system must have a means to enric
sample of "not normal" cases to a degre
it is practical for a pattern recognition
tion to analyze and select a small num
cells, if any for final manual evalu
3 The system should be capable of pro
at least 120 cases per hour With a 1
day 230 days per year this would am

331,200 patients. The system should not select more than 16,560 of these for human review or 72 per day

4. The hardware cost of the system in production form should not exceed $250 000.

The discussion which follows is based on these practical considerations and requirements It is felt that the criteria stated above are reasonable. There is no question that the requirements of no-missed-positives (to the extent measurable by present procedures) and the sufficient enrichment of final samples for human review present major technical challenges. In the author' opinion, the $250,000 maximum production cost is necessary for reasons beyond the financial advantages over human review of 331,200 specimens per year at a cost of $5 00 each, which is the effort such a machine should replace. Experience in the design and production of automated systems for medicine has shown that systems which exceed this cost and corresponding complexity tend to defeat their own purpose by complicating the "trained manpower problem and the investment required by medical institutions and laboratories discourages acceptance

Whatever parameters are measured to determine normal versus abnormal, it will be essential that threshold settings in the machine be such that the emphasis is *on rejection of normal cells from the specimen rather than passing of clearly abnormal cells* The threshold settings must not be set to pass only obvious abnormals for the sake of rapid enrichment of the final sample to screening manually The enrichment must be secondary to assuring that borderline abnormals are not rejected with the normal population This is a consideration that can be easily overlooked with disastrous results The simultaneous solution of both problems, no-missed-positives and sufficient enrichment of final sample, will likely require approaches more sophisticated than one-pass measurement of a single parameter Two design approaches appropriate for flowthrough systems are suggested below The first, multi-parameter selection, places emphasis on sharpening the discrimination between normal and abnormal cells the second, multi-cycle enrichment, may be necessary if the no-missed-positives criterion is not compromised The *no-missed-positives* criterion will undoubtedly determine acceptability The *enrichment* characteristics of an automated system will determine *practicality*

Multi-Parameter Prescreening

At the present time there is not sufficient evidence to indicate that any of the parameters now being measured by automated prescreening machines are by themselves sufficiently accurate and selective to meet the "no-missed-positives and enrichment requirements It is the author' opinion that the selection criteria for separating normal from abnormal cells must involve multi-parameter measurements to be both acceptable and practical Cell volume measurements by the Coulter technique and measurement of nuclear material with fluorescent stains have been implemented in flowthrough systems. There appear to be no major technical or engineering barriers to connecting such units in various serial and parallel combinations A multi-parameter selection configuration would require that a cell must meet normal" criteria in two or more parameters before *it is rejected from the sample which is processed by subsequent stages* It is essential to keep in mind that the thresholds of high-confidence normal criteria and high-confidence abnormal criteria are not the same The former must be used in a prescreening system The problems of adequate flow rate and enrichment can be improved as well with multi-parameter selection. The greatest benefit in these cases can be achieved by recycling the selected sample through successive stages This will be discussed in the next section.

Figure 6 shows a configuration using two selection parameters which requires that a normal cell pass both parameter 1 *AND* parameter 2 threshold criteria. *Note that the emphasis in this configuration is on accurate selection of normal cells* rather than selection of abnormal cells If it is assumed that the abnormal sample selected from the specimen is small compared to the total specimen, then each of the units P1 and P2 shown in Figure 6 must be capable of comparable flow rates.

Regardless of the average error which can be ascribed to any particular threshold setting for a single processing unit such as P1 *there is always the random or transient error which occurs in any selection process involving physical objects*. This is particularly true of biological specimens The configuration shown in Figure 6 is a coincidence circuit which provides protection against random or artefact error that would allow positive cells to be rejected as normal This scheme can be used when parameters measured by each unit are the same or different Many other obvious combinations of similar or dissimilar units can be connected in this serial-parallel configuration to achieve the desired result

Multi-Cycle Enrichment

The other consideration associated with each parameter measurement is the enrichment factor This is simply the percentage of total cells retained abnormal by a particular parameter measurement and threshold setting It is the author opinion that substantial enrichment of a specimen using the cell volume and nuclear mass

Dealing with raw biological material for scanning it would be more prudent at this moment in time to aim for the identification of signals than separation of cell samples into malignant and non-malignant. The overlap is too great. However many parameters can be used in the pre-screening templates. Success is more likely and more confidently achieved if the final arbiter is the human eye, viewing the signal

As experience is gained in parameter selection and scanning techniques, then the ultimate separation will be welcomed as a bonus.

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DR. WATTENBURG

System Design Approaches for Automated Cytology Prescreening

It is the author's opinion that any automated or semiautomated cytology prescreening system designed today which relies primarily on the measurement of a single parameter such as cell volume, DNA RNA mass, etc., as these parameters are now being measured, will have great difficulty competing with trained cytotechnologists in a *production* screening environment. Furthermore the primary requirement of no "missed positives" and the practical requirements of processing rate and efficiency of selection (enrichment of final specimen for manual screening) raise grave doubts as to the ability of a "single cycle" system meeting all of these requirements Consequently *it is proposed* that any design and implementation effort toward achieving a practical automated system give serious consideration to the use of a multiple parameter multi-cycle system as described herein

The Problem

Wied has described the problem in its essential form

1 The prescreening system must not miss any present tumor cells, i e no 'missed positives'
2 The system must have a means to enrich the sample of 'not normal' cases to a degree that it is practical for a pattern recognition operation to analyze and select a small number of cells, *if any* for final manual evaluation
3 The system should be capable of processing at least 120 cases per hour With a *12 hour day* 230 days per year this would amount to

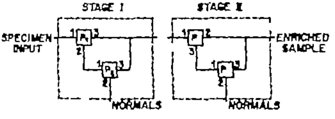

FIG 7 Schematic drawing showing multicycle enrichment of the abnormal sample

abnormal cells are noted in stage I similar activity would be expected in subsequent stages When electronic equipment such as that used in cell volume or nuclear mass measurements is available, this is easily implemented by monitoring the activity of the threshold circuits Some failsafe circuitry of this sort is necessary in any practical automated system that is expected to handle large numbers of specimens on production basis The specimen processing time is small, and so is the probability of detecting anomalous behavior before large numbers of specimens have been processed Again, there are many possible ways of providing error checks by monitoring the activity of the parameter measurement units in configurations such as shown in Figures 6 and 7

Design and Implementation Philosophy

In an automation project immediate and substantial improvement through automation becomes primary consideration independent of cost When the prospect exists that the machine can measure the parameters or make determinations which cannot be done manually *with the same degree of relevance to the final decision* This may be possible in cytology if several of the parameters now measured qualitatively by the trained cytologist are utilized by machine more consistent and quantitative manner for selection of abnormal cells It is the author' opinion that any machine designed today which relies primarily on the measurement of single parameter will not achieve such an improvement or breakthrough This is the reason for the suggestions of multi-parameter and multi-cycle configurations as described above The history of automation projects such as this has shown that significant new techniques and information most often come as a consequence of the efforts to automate the best available solutions to difficult problem *with the imposed requirement that the automated solution be as good or better than manual procedure alone* Excessive time and effort spent on automating known solutions to carefully selected problems (specimens in this case) instead of facing the realities of the typical case seldom forces breakthroughs which make practical production systems possible This has been the modus operandi of those who have produced the most significant automation systems in existence today If this approach is accepted, it is easy to recognize where effort and emphasis must be placed now to achieve any significant automation in cytology This is most easily demonstrated by remembering that all of this discussion assumes the existence of clean specimens which can be processed by the techniques discussed above The whole discussion above or any other approaches are academic until adequate means can be developed to provide specimens of uniform characteristics and sufficiently free of artefacts to be handled by automated measurement equipment

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DR SCHMIDT WEINMAR

Special Consideration of Optical Diffraction

Prescreening with Laser Light

Prescreening will accomplish a rapid and economic characterization of cell samples It may be followed by the cytopathologist examination or by automated cell identification At the level of prescreening, an automated device may miss single positive events, for example the occurrence of single malignant cells But the results of measurements done on large number of cells (representative sample) will allow the specimens to be classified with high degree of certainty the number of "false alarms should possibly be low "

The sample may consist of cells which are either suspended and carried to the measuring spot by flow system or are simply presented on a microscopic slide The cells may be fixed and stained, but methods which would allow observations in vivo will be included in the considerations The following discussion concerns optical probing methods as applied to prescreening What advantage may be gained by using a laser as light source?

If compared with thermal light sources or with the cathode ray tube, the laser distinguishes measurements as now implemented will require a multi-cycle system in order to achieve the required enrichment percentage and simultaneously control the selection error (missed positives). This problem is usually solved in flow systems by using two or more successive stages such that the enrichment is increased in two or more serial stages while the random selection error (missed positives) is controlled with coincidence circuits within stages as shown in Figure 6.

Figure 7 is a block diagram of a two stage system. Each successive stage is required to handle a lower flow rate, which typically allows better selection and a lower error per stage. Multi-cycle systems such as this are almost always adjusted so that the early cycles have wider "selection windows" than the final cycles which are adjusted for sharpest discrimination because of the differences in flow rates. But still, it matters not how much enrichment is achieved if positives are being rejected with the normal population. Hence each of the stages contains within it the "coincidence configuration" shown in Figure 6. It is obvious that a great variety of combinations of multiparameter and multi-cycle configurations is possible in a continuous flow measurement and selection system. The particular configuration appropriate for a given specimen selection and enrichment process depend on the characteristics of the individual units, their flow capacities, and discrimination characteristics.

Optical Scanning

Direct optical scanning and recognition of morphological characteristics does not lend itself so easily to a multi-cycle configuration as described above. The state-of-the-art in this field is such that this technique is best used as a final stage in a prescreening system. It is likely that cells to be scanned will have to be fixed in some way such that they are immobile and enclosed in an environment of uniform optical characteristics by the time they reach the optical scanner. This will likely require specimen preparation incompatible with the cell volume and nuclear mass measurements performed on liquid specimens.

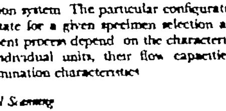

FIG 6. Schematic drawing showing multiparameter selection of normal cells for each unit (1) sample input (2) normal output, (3) abnormal output

It is the author's opinion that specimens must be enriched by previous selection stages to the degree that no more than 100 cells must be scanned and analyzed by direct optical means if significant morphological characteristics must be extracted and analyzed. High speed scanner-computer combinations of reasonable cost ($150,000 to $300 000) can perform such tasks in the order of one second per cell, at best. The requirement for processing 100 specimens per hour allows 30 seconds per specimen, which is why the 100 cell maximum is reasonable even taking into account expected improvements in this technology in the next few years. Performing less sophisticated scanning and recognition operations at a higher rate begs the question "Why use an optical scanner?" Morphological characteristics are essential in the decision process and optical recognition is the only means available.

However practical implementation of a production optical scanning technique with automatic specimen preparation will prove to be one of the most difficult tasks of those proposed to date. Automatic preparation of specimens for an optical scanner has often proven to be every bit as difficult as the construction of the scanner electronics and computer programs to control the scanner and analyze the optical data. There is every reason at present to believe that this will be the case for an automated cytology system.

The complex pattern recognition operation performed by the trained human eye goes far beyond any combination of all the measurement techniques mentioned above. Hence, there is no question that any acceptable cytology system will *be a prescreening system* for the foreseeable future. Just the subtle morphologic characteristics easily recognized by the human eye far exceed the capabilities of present day pattern recognition *systems if these systems were called upon to perform such tasks in an acceptable and practical production environment at reasonable cost*

Failsafe Design

Another consideration often overlooked by designers in systems which must perform tasks as critical and important as this one, is the requirement for built-in failsafe procedures to prevent short term anomalous operation. The danger in a cytology system as described above is that an undetected failure may occur which passes abnormal cells as normal. In flowthrough systems as shown in Figures 6 and 7 there is a technique which can be used to safeguard against such undetected failures. This involves an additional "coincidence circuit" which expects simultaneous activity in all circuits when it finds activity in any one or more circuits. For instance if err

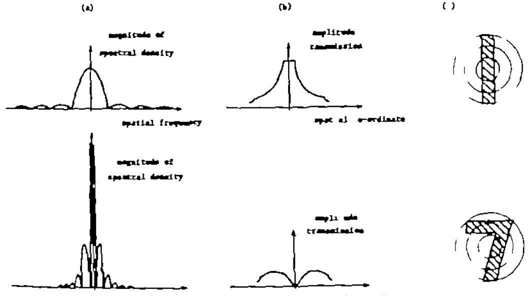

Fig 8 (a) magnitude of the spectral density (b) corresponding object transmission functions These transmission functions are the *mean* taken over all equally probable orientations of the transmission functions of the symbols 1 and 7 shown in (c) A real filter acting on the magnitude of the spectral density can in this way distinguish between objects, whatever their positions and orientations in the object plane

If complex matched filters are used, the information on the position of the detected object also retained in the transmitted light[10] Further complex filters are sensitive to the structure of the object in three dimensions They possess, therefore, much higher discriminatory power than other filters

Although this is very favorable indeed for the potential application of such filters to pattern recognition problems, one must consider how much of the high discriminatory power must be relinquished so that complex filters can cope with *distributed parameters* as they occur in biologic specimens For instance, how sensitive are such filters to the unavoidable distributions in the scale orientation, position (geometric parameters) and shape of the cells of a given population At first glance it may seem that any potential advantage of complex filters must be abandoned if such filters are to be applied to objects which have statistically fluctuating rather than distinct parameters. Fortunately this problem an (for prescreening purposes) be solved in way other than by reducing the high discriminatory power of such filters

There is complex filter that matches any given structure of object in three dimensions If such a structure is, in fact, relevant to the considered diagnostic problem, and if the matched filter is highly selective for this characteristic structure, the occurrence of only few positive events detected from the transmitted signal can be considered as sufficient indication of the searched for cytopathologic symptom, g malignancy It is convenient to assume that within a representative sample of cells there will be few that match the filter specification, if the specimen satisfies the given condition of classification.

Variations of geometric parameters of the object may be compensated for by external means For instance number of matched filters of different spatial orientations may be applied sequentially to the sample Also, the scale of the object can be changed by optical methods Suitable techniques to make complex filters more adaptive and less sensitive to the geometric parameters of the object[14] are being studied. Theoretical work on the sensitivity of matched complex filters to parameter variations has been recently reported

Matched complex filters, which are sufficiently insensitive to geometrical parameters of the cell to be screened, perform the reduction of the data on the object at almost the same stage of the data processing as does the computer The remaining problem is to computer-synthesize filter that matches the "synthetic object"[17] or in other words, that contains the intersecting set of data which is common to all objects of a given classification.

itself by the high radiance, the high directionality and the high coherence in space and time, of its output.[4] High power pulses of very short duration may be generated. In addition, the laser can be run as a scanlaser[18] or the light emerging from an ordinary laser may be deflected in such a way that high-resolution, high-speed optical beam deflection is performed. The scanning rate may be as high as 10^4 deflections per second.[8] Since at the measuring spot the intensity from a laser is very high, such a device may be applied effectively for rapid sampling of microscopic specimens or optical parallel probing. Herewith the high radiance and directionality of the laser light rather than its coherence would be used.

Measurement of Optical Diffraction

The high radiation of the laser output is of considerable interest in optical diffraction experiments because a small range in direction of the incident light is essential for any qualifying interpretation of the light scattering data. For instance lasers have been used to study the differential light scattering by viable bacterial cells[17] and to measure the spatial spectral power density of the optical transmittance of metaphase cell spreads.[14] In these experiments the intensity of the scattered radiation was measured and no attention was given to the phase of the diffracted waves; therefore, one-half of the total information on the structure of the scatterer is not evaluated.

However, conclusive information on the structure of the diffracting objects may be obtained from the intensity of the diffracted light.[7] This would occur, for instance, in cases where periodic arrangements of identical unit cells are studied or where some pre-knowledge of the physical shape or the optical constants of the object exists. In particular, knowledge of symmetry properties of the diffracting object is beneficial.[1] However, the more irregular the object is, the more ambiguous the interpretation of the diffraction intensity data becomes.

But must we return to the space-domain in order to perform prescreening? The intensity of the diffracted light may, by itself, tell something very useful to cytodiagnosis. For instance, if everywhere in the cytoplasm a similarly structured pattern (of suitable spatial frequencies) arises, having some relevance to malignancy, the measurement of the intensity (rather than intensity and phase) of the diffracted light would be entirely sufficient.

Filtering

Light diffraction can be effectively employed for optical filtering. Since at an early stage of the data processing, an optical filter reduces the data by physical means, the feasibility of filter techniques as applied to prescreening has to be considered. In particular, how does the sensitivity of such filters with respect to the position, scale and orientation of the objects limit their application to samples of cells, in which the position, scale and orientation is randomly distributed? The great potential of suitable optical filters for rapid cell identification has been demonstrated recently.[9,1]

A. Real Filters

A real filter is a transparency placed in some plane of an optical arrangement in order to attenuate the amplitude of the transmitted light, thereby not changing the phase. Such a filter can be realized if the filter transmission function is sufficiently smooth.[3]

A real filter may be placed in the focal plane of the objective lens where it is capable of detecting cells of a certain pattern, irrespective of their position, scale and orientation. Within the field of view such a filter may even be used to detect simultaneously a group of cells, the averaged pattern of which matches the filter. This is demonstrated in Figure 8. Taken over all equally probable orientations, the filters shown match the average transparency of the symbols 1 and 7 respectively. Similar considerations may be applied to match a filter to a size distribution of the cells. If a real filter is placed in the back focal plane of the objective lens and if the phase of the light is not detected, the filter is largely insensitive to the position, orientation and scale of the cells. It can, however, retain enough discriminatory power to be useful for cytodiagnostic applications.

B. Complex Filters

Complex filters change both amplitude and phase of the transmitted light; they may be designed to yield any arbitrary complex transparency[18] if their transmission function is sufficiently smooth. Particularly attractive is the possibility of computer-synthesizing such filters. These filters can perform a wide range of linear operations on the amplitude and phase of light distribution in the filter plane, for example operations of two-dimensional autocorrelation, crosscorrelation, convolution, integration, differentiation, etc. Hence, complex filters are of considerable interest to pattern recognition.

Figure 9 shows, as an example, the higher discriminatory power of a complex filter as compared to a real filter. A real filter cannot conclusively detect intracellular asymmetries,[17] but morphologic features which lack central symmetry may be essential for cytodiagnosis.

Discussion

Drs Bartels, Bahr and Wied

The fact that Dr Diamond used truncated distribution function for his study presents difficulty for attempts in estimating the underlying parent distribution. It would be quite valuable and undoubtedly very informative for the reader if Dr Diamond could be so kind as to prepare for us, on the basis of his raw data, the frequency of occurrence plot of large nuclei for normal samples and abnormal samples, and how these values are distributed in each diameter class among different patients This, combined with data on the expected frequencies of occurrence their variance and confidence limits, would offer much better basis for an assessment of the suggested method, and allow a better projection of an expected error rate

Dr Shuster and his co-workers may be interested in recognition algorithms recently developed by us Here short series of gray values—typically from five to nine values, are measured at 0 5 μ intervals through the nucleus of cell For each of a number of given cell types, the covariance matrix for such sequence of gray values is then estimated on training sets of approximately 100 cells each The observed value sequence in an unknown cell can then be assigned to cell type on the basis of maximum likelihood The method is independent of staining intensity when normalized for the average gray value per sequence It leads to very high likelihoods for recognition in cells with usually noticeable differences of chromatin granule distribution, and to acceptable likelihoods cases where visual observation does not allow an unequivocal distinction Computation could be done on special minicomputer and is not prohibitively complex

Dr Shuster and co authors seem to see the analyses performed in the TICAS project as essentially one parameter approach This is correct only in the sense that gray values are measured exclusively In the sense that under parameter" an information carrying dimension is understood, the master program of the TICAS project routinely employs from 60 to 90 dimensions in its supervised learning section, and after suitable feature selection approximately 10 dimensions in the nonsupervised learning section Recent development of program section which utilizes the covariance of large two-dimensionally extended gray value arrays for recognition of very minor changes in the texture of cell images typically employs 400-dimensional feature vector

Dr Bosso

I wonder if Dr Husain could provide us with the signal-to-noise ratio of the Quantimet apparatus with which he has performed his interesting studies

Dr Cram

Approaches to prescreening have been examined in the papers of this section with the thought in mind that many more cytotechnicians are needed to be able to screen all women over 20 in the United States on an annual basis With present prescreening capabilities, only minority of such women get Papanicolaou smear analysis Prescreening, as the cytotechnician performs it, is time-consuming and tedious operation especially as Tolles points out, when 92 to 96 per cent of the samples are negative

The consensus of the prescreening papers appears to be that no one knows how best to alleviate the problem Of the ten papers in this section, those by Husain, Rosenberg *et al* Schmidt Wemmar Heasley and Diamond are, respectively concerned with multiple parameter image analysis, decision theory optical probing for pattern recognition, and various transport systems for presenting samples to such sophisticated machines Their prescreening approach requires that subtle morphologic characteristics, easily recognized by the human eye-brain combination, be analyzed automatically by machine I agree with Wittenburg that direct optical scanning is best used as final stage for suspicious cells and not as high-speed prescreening device Shuster *et al* points out that, because cancer detection by cytologic techniques is based on visual criteria, it has been reasonable to build machines designed to mimic the visual observations Such an approach is acceptable if the criteria used by the cytotechnician in making decision can be more effectively evaluated by machine, resulting greater degree of accuracy This has not been the result to date

It is my intention to discuss ideas presented the other five papers and to see how they might be successfully combined to give prescreening device that is not too complex to be used in community hospital and that would also be big er to multiphasic screening centers currently being proposed to improve health care delivery in this country

It seems reasonable therefore to take advantage of Kamentsky and Hulett and Herzenberg ideas to establish some new directions Kamentsky and Hulett and Herzenberg discuss the advantages and many parameters that can be measured using flow [illegible] Kamentsky

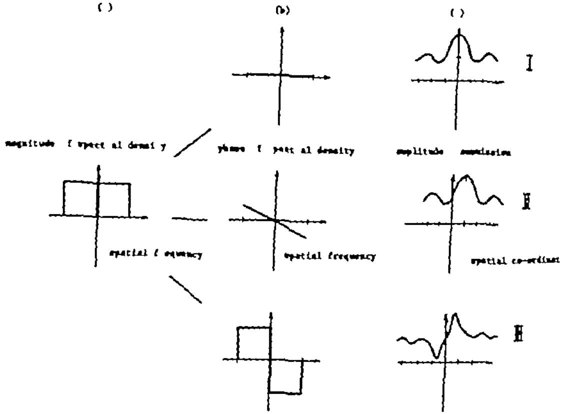

Fig 9 (a) magnitude (b) phase of the spectral density of three different object transmission functions, shown in (c) A real filter acts on the magnitude only of the signal and can therefore not distinguish between objects I II and III In contrast, a complex filter acts on both amplitude and phase of the signal and can, therefore, distinguish between objects I *II and III*

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1 The instrument should be designed specifically as prescreening instrument to differentiate between "normal" and non-normal" cells

2. The instrument should classify as non-normal" cellular changes due to inflammation, hyperplasias, atypias, and, of course, frank malignancies

3 The instrument should incorporate a flow system designed to transport cells rapidly so that the instrument can examine more cells in shorter time than are generally reviewed manually by conventional microscopic examination of smears

4 A high resolution focused optical system should be used to examine the flowing cells in order to measure precisely the bsorption char acteristics and morphologic structures of the individual cells

5 An ultraviolet light system should be used in order to avoid the inaccuracies and nonreproducibility introduced by staining the cells

6. The instrument should be capable of rendering decisions accurately t rate considerably greater than is possible with manual cytologic examination

7 I view of the wide variety of cellular changes to be encountered in cytologic screening, the instrument should have the ability to measure and compute number of parameters based upon variety of cytologic criteria

By following the general philosophy described above, the author and other members of the staff of Nuclear Research Associates, Inc have developed an instrument known as the NRA 2008 Cytoscreener which is an operational optical scanning and digital computing system capable of rapid cytologic prescreening [A description of this instrument may be found in the *Journal of the Association for the Advancement of Medical Instrumentation* (4:1–6 1970)]

Dr Galli

Discussion on prescreening involves in our opinion tw main points

1) conditions to be satisfied by prescreening system in order to be acceptable and practical
2) which (if any) of the methods proposed till now fulfils (or is expected to fulfil in the future) the requirements listed in point 1)

Concerning acceptability single prerequisite is strictly necessary *the system must not produce false negatives* (at least at higher rate than manual prescreening (manual and automatic sensitivity indices must be comparable)

I the practical evaluation of the system, considerations of cost, organization and staffing become important The system is to be considered *recognizer f normal samples* It becomes practical when the cost corresponding to each normal sample recognized is competitive with the cost of normal sample recognized by man (here the cost is meant in its broader sense) The ratio of normal samples recognized to normal samples analyzed gives the specificity index It is not necessary to put limit priori to this value there is no reason to reject priori simple and inexpensive hypothetical system capable of recognizing say 50 percent of normal samples. Speed considerations have to be included in the general frame of the cost problem as w can always obtain, if necessary a given speed by using more sub-systems in parallel

An unquestionable advantage would be that of using for screening the same object utilized for prescreening This condition, however is not in general essential, as different samples of the same person can be utilized

As far as regards point 2) no system so far proposed is capable of fulfilling the requirements listed The problems to be solved are basically of two types

) convenient cell presentation to the machine, so as to avoid the negative influence of artefact and clusters
b) automatic measurement of set of parameters for each cell capable of selecting normal cells with the required reliability

In our opinion *flow systems* based on *multiple parameter* determination seem at this moment to be the most promising High resolution scanners using pattern recognition techniques are expensive and still have problems of cell isolation. Their use seems more appropriate in the next phase of screening, where the quality and quantity of morphological information which the method is potentially capable of giving can best be utilized

Dr Holm

I agree with many of the points made by the various authors in the section Approaches to *Prescreening but would like to take issue with* some of their comments and suggest some areas for greater emphasis or new thought

Representative Sampling

Tolles, Husain, and Shuster *et al* have mentioned the sampling problem, and it seems that at present about 15 per cent of the "real cervical cancer cases are missed by standard cytology and that most of this is attributed to the sample not containing recognizable malignant cells If this is true, then substantial gain in detection rate could be effected by designing an instrument, technique or training procedures that would improv sample collection Indeed, this might well be the most profitable area in which to expend major effort. This problem should for rejecting the slide smear as a means of cell presentation to a machine Cell positioning and transport problems are eliminated in Kamentsky's flow system. Using a flow system, Hulett has made several fluorescence measurements on different systems cell separation and detection of "relatively small" histocompatibility differences between cells and sera establish this approach as a potential prescreening technique. The critical problem becomes that of determining biologically useful parameter(s) for the machine to measure Hulett makes several very good suggestions such as enzyme activity and lipid content as acceptable criteria that would indicate the pathologic condition of cells. Metabolic activity and surface charge density are also suitable for machine monitoring changes in all of these parameters have been associated with theories of malignancy

Additional suggestions and requirements for any prescreening machine were examined by Wattenburg Tolles, and Shuster *et al* Wattenburg proposes a system designed for multi-cycle enrichment of abnormal cells by placing emphasis on the rejection of normal cells. Because normal cells are more readily defined than are abnormal cells, this approach is valuable in that it should assure that borderline abnormal cells would not be rejected with the normal cells. Tolles presents a very realistic review of prescreening concepts and performance in routine and experimental cytologic screening His emphasis on obtaining a high throughput factor is important but cannot be considered crucial in a tradeoff against being able to screen all women over 20 Shuster *et al* suggest, and rightly so, that a prescreening machine needs to reduce the number of normal cells by only 60 to 70 percent to decrease tedious prescreening substantially and increase sample handling capabilities such a machine need not involve a complex pattern recognition scheme Rosenberg *et al* as well as Shuster *et al* emphasize that two-parameter prescreening would be desirable to eliminate "overlap between critical criteria common to a small percent of both normal and abnormal cells this is important and is consistent with high-speed measurements

Problems of sample collection and preparation were mentioned by Hulett, Wattenburg Shuster *et al.*, and others who agreed that these problems must be given considerably more attention if new approaches such as those mentioned above are to succeed. The problems of cell preparation for flow systems were compared to those already solved for fixing and staining cells on slides (Hulett) This is rather optimistic, although I believe that it is reasonable and possible to develop procedures that will give machine-specific samples.

In summary criteria of malignancy other than conventional morphology are being suggested and investigated. Theoretically any change that alters a measurable cell parameter may be amenable to machine measurement at high speeds (10^4 to 10^5 cells/minute) and high precision, hence, offering much simpler means of prescreening cancerous cells than do machines that are designed to make pattern recognition judgements. These more sophisticated (and slower) machines could then be economically used for examining only suspect samples.

DR. EHRLICH

Re Rapid Examination of Unstained Cells in a Flow System

The concept of being able to use an instrument to examine cytologic specimens automatically or semi-automatically has been considered by many investigators for approximately twenty years. It was generally felt that the state-of-the-art in optical scanning and digital computing provided sufficient technology to make the development of such an instrument possible However most of the attempts made to date have accepted artificial constraints upon the instrument designer because the investigators hesitated to adopt unconventional guidelines for their efforts. For example consider the fact that the vast majority of these investigators decided to accommodate to the examination of conventional ("Pap") smears, a decision which imposes inordinate constraints in the design of an automated instrument for rapid cell scanning

The most troublesome of these constraints have been control of focus, overlapping of cells, the presence of non-cellular debris, the unevenness of the smears, and the presence of clusters of small cells Even if those problems associated with smear preparation could be overcome one would still be faced by the additional difficulty introduced by the non-stoichiometric nature of the staining reaction and the non-reproducibility inherent in the Papanicolaou and other staining techniques Unfortunately those few staining systems which are stoichiometric and selective for specific cellular constituents are difficult to control and to standardize

In view of these smearing and staining difficulties, we felt it would be desirable to avoid the conventional "Pap" smear as the method of presentation of the cells to the instrument. Consequently we approached the development of a prescreening instrument without imposing this requirement upon the instrument designer

After careful review and consideration of the work of the leading investigators in the field, we concluded that the most logical and reasonable approach to instrument design would involve the following objectives be emphasized that the instrument proposed could only be used on the 75 per cent of the population that is not now being examined It is important to realize that there are many ways of improving the present situation and that the requirements of the system must be integrated with the way it is employed

Wattenburg also mentions the failsafe requirement. That is important, of course, but there are other approaches in which a standard (probably synthetic) is inserted with the sample and is required to be measured properly along with the sample I this technique, the complete system is monitored all the time

In conclusion, after 15 years of intensive effort in scanning systems, they still do not appear t offer any real hope of satisfying the need of prescreening device No single parameter system that has been developed at this time will solve the identification problem, but multiparameter flow systems have enough promise t warrant further investigation The ideal would be to find one or more parameters suitable for automation and specific for malignancy

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Dr Ishiyama

Human eyes recognize patterns as whole, but machine does so by scanning Again, human eyes catch patterns qualitatively or semi-quantitatively but machine only quantitatively Therefore in order to let machine recognize cell, devices for recognizing an individual cell as single cell are to be elaborated at the first step Thereafter an automated measurement system is to be established As for the parameters of automated measurement, I believe that both the geometry (measurement of area) and densitometry (measurement of optical density) are important I am proceeding on automated cell measurements using a prototype apparatus called Photomicroscanner" which our research group invented two years ago I mentioned the temporary results in the session Classical Quantitative Measures" in this Symposium

I believe that machine should simulate discrimination way of human eyes because here is no discriminator of cancer cells superior to human eyes at the present stage On the contrary neither flow systems nor estimation of DNA content are similar to the way of human eyes, and these methods might not overcome human eyes in discriminative power For the above-mentioned reason I myself adopted slide method, though this method also has many difficulties to be resolved Our devices for automatic prescreening have been developed for several years This apparatus, the Automatic Cytoscreener is still prototype and has many problems to be improved, I preparation of specimens, the light source, resolving power of the lens system, cytodiagnostic criteria, cost/performance relationship and so on However this machine can recognize an individual cell as single cell, and measure and process areas automatically Moreover function of measuring and processing optical density according to the classical formula ill be added in the near future At the present stag our cytophotometric apparatus, Photomicroscanner" measures optical density the classical sense exactly

As far as automated cytodiagnosis aims at the prescreening step it is desirable that errors of the first kind (false negatives) should be as few as human screeners might err due to fatigue or careless examination But at the present status of engineering level it is impossible to hope that any automated methods may be able to recognize patterns as skillfully as human screeners do. Then, is it meaningless to elaborat upon devices which substitute human tasks for prescreening cancer cells? No, I believe efforts are necessary because of the present miserable situation where no prescreening machines are in practical use I considering the bove-mentioned status, research workers should not project tentative and dexterous apparatus A prototype apparatus should be basic one for future improvement which could acquire and process exact information upon which the talented cytotechnologists may agree This kind of apparatus would not apply to automated prescreening at present because of insufficient ability of pattern recognition but it can be applied to the precise automatic measurement of any cells which cytotechnologists recognize under he microscope Thereby precise quantitative cytodiagnosis is practical enough under present circumstances and accurate automatic prescreening will be expected in the near future by steady efforts for improvement

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Dr Melamed

The [illegible] prescreening is useful only because it indicates that screening can and should be carried out in stages Good laboratories already do this with a hierarchy of cytotechnologists certainly be worked on regardless of the prescreening outcome.

Directions of Research

The real problem in cancer diagnosis is that there is no known definitive test to indicate malignancy with a very high probability. This fact was presented by most of the authors in one form or another, and lists of parameters that have been tried are provided by Shuster *et al* and Husain. Attempts to mimic the pathologist are numerous and well documented. They might even succeed, but prognosis for mimicry does not look very encouraging at this time. This is because the pathologist makes his decision based upon a number of factors, each of which has a fairly low probability of specifically indicating malignancy. It would be very fortuitous if morphological changes now being examined by pathologists are the most significant changes that occur in the transformation of normal to malignant cells. Therefore, it seems reasonable to search for changes that are different from those now being examined. Hulett and Shuster *et al* suggest monitoring enzyme activity or changes in carbohydrates or lipid content. Use of radioactive tracers with exfoliated cell cultures was also mentioned as a possible prescreening technique, and this would be acceptable. However, the current attitude about radiation would prohibit ingesting radioactivity for any prescreening method, but the use of stable isotopes followed by activation analysis might be suitable. Activation analysis has very high sensitivity for certain elements and is amenable to automation. Also, there may be unknown electrical properties of cells that could be used. There is no indication in any of the papers that the search for new and more specific parameters has been more than superficial.

Perhaps the most important effect of this Symposium will be the realization that a reorientation of priorities should be made. In this vein, I would like to propose that the effort in eye mimicry be decreased in favor of looking for more fundamental and as yet unobserved phenomena in a gamble that it will offer the best long-range hope. This would amount to some retrenching and admission that automatic prescreening is not in the immediate future. It would also mean providing support for what might be considered basic research or high risk gambles on novel approaches. As an interim solution, it would be advisable to concentrate some of the effort on developing multiparameter flow systems and tests for specific forms of malignancy with good treatment prognosis and high incidence rates. Since cervical, rectal, breast, and skin cancers fit this condition, it would be wise to concentrate on these types. Combining the tests of Rosenberg *et al* and Diamond might produce a multiparameter instrument that would be suitable for prescreening cervical cancers. Some experiments reported by Holleb indicate that Gold has found a specific for rectal cancer that may be suitable for automatic prescreening. It is not obvious how automated cytology could be applied to breast or skin cancer diagnosis, but it might be possible to analyze secretions from the breast and scrapings from the skin. However, prescreening by physical examination or by thermography, where small temperature differences are measured, might be the better methods. Since some of the most sophisticated technology of our times has been applied to scanning systems with minimal results, why not seriously consider new approaches?

Requirements of Prescreening Instruments

Wattenburg quoted Wied in outlining the characteristics of a prescreening device: "The prescreening system must not miss any present tumor cells, i.e., 'no missed positives,' should enrich the sample in 'not normal' cells, should process 2 cases per minute, and select the proper 5 per cent of not normal cases, and should cost no more than $250 000." These are certainly reasonable requirements but should not be considered more than guide lines. For example, one might be willing to make a trade-off between cost and throughput. I think the choice of words relating to the "no missed positives" concept is poor because it implies that none is missed under present conditions. Wattenburg later qualifies it to mean no more than at present. Then a more accurate criterion would be: "The prescreening system must result in more of the malignant cases being diagnosed than at present." Wattenburg's statement does not put the present method in perspective, where 15 per cent appear to be missed by the combination of sampling and slide examination.

There is another way of looking at the "no missed positive" concept. If only about 25 per cent of the female population are being examined at present and they are reasonably representative of the population, then up to 75 per cent of the positives are now being missed. Therefore it would be worthwhile to implement a system that would examine only the other 75 per cent even if the system had a 50 per cent miss rate for detecting abnormal cells but recognized and disposed of normal cells with a 99 per cent probability. With this proposed enrichment factor for abnormal cells, the work load would only increase about 10 per cent on the technician, but the number of detected malignancies would more than double. Here again is a trade-off, in this case an enrichment factor for missed positives. It should be emphasized that the instrument proposed would only be used on the 75 per cent of the population that is not now being examined. It is important to realize that there are many ways of improving the present situation and that the requirements of the system must be integrated with the way it is employed.

Wattenburg also mentions the fail-safe requirement. That is important, of course, but there are other approaches in which a standard (probably synthetic) is inserted with the sample and required to be measured properly along with the sample. In this technique the complete system is monitored all the time.

In conclusion, after 15 years of intensive effort in scanning systems they still do not appear to offer any real hope of satisfying the need of a prescreening device. No single-parameter system that has been developed at this time will solve the identification problem, but multiparameter flow systems have enough promise to warrant further investigation. The ideal would be to find one or more parameters suitable for automation and specific for malignancy.

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DR. ISHIYAMA

Human eyes recognize patterns as a whole but a machine does so by scanning. Again, human eyes catch patterns qualitatively or semi-quantitatively but a machine only quantitatively. Therefore in order to let a machine recognize a cell, devices for recognizing an individual cell as a single cell are to be elaborated as the first step. Thereafter an automated measurement system is to be established. As for the parameters of automated measurement, I believe that both the geometry (measurement of area) and densitometry (measurement of optical density) are important. I am proceeding on automated cell measurements using a prototype apparatus called "Photomicroscanner" which our research group invented two years ago. I mentioned the temporary results in the session "Classical Quantitative Measures" in this Symposium.

I believe that a machine should simulate discriminative ways of human eyes because there is no discriminator of cancer cells superior to human eyes at the present stage. On the contrary, neither flow systems nor estimation of DNA content are similar to the way of human eyes, and these methods might not overcome human eyes in discriminative power. For the above-mentioned reason I myself adopted a slide method, though this method also has many difficulties to be resolved. Our devices for automatic prescreening has been developed for several years. This apparatus, the "Automatic Cytoscreener," is still a prototype and has many problems to be improved in preparation of specimens, the light source, resolving power of the lens system, cytodiagnostic criteria, cost performance relationship and so on. However this machine can recognize an individual cell as a single cell and measure and process areas automatically. Moreover a function of measuring and processing optical density according to the basic formula will be added in the near future. At the present stage our cytophotometric apparatus "Photomicroscanner" measures optical density in the physical sense exactly.

As far as automated cytodiagnosis aims at the prescreening step, it is desirable that errors of the first kind (false negatives) should be as few as human screeners might err due to fatigue or careless examination. But at the present status of engineering level it is impossible to hope that any automated methods may be able to recognize patterns as skillfully as human screeners do. Then, is it meaningless to elaborate upon devices which substitute human task for prescreening cancer cells? No, I believe efforts are necessary because of the present miserable situation where no prescreening machines are in practical use. In considering the above mentioned status, research workers should not project a tentative and dexterous apparatus. A prototype apparatus should be a basic one for future improvement which could acquire and process exact information upon which the talented cytotechnologists may agree. This kind of apparatus would not apply to automated prescreening at present because of insufficient ability of pattern recognition but can be applied to the precise automatic measurement of any cells which cytotechnologists recognize under the microscope. Thereby precise quantitative cytodiagnosis is practical enough under present circumstances, and accurate automatic prescreening will be expected in the near future by steady efforts for improvement.

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DR. MELAMED

The misnomer "prescreening" is useful only because it indicates that screening can and should be carried out in stages. Good laboratories already do this with a hierarchy of cytotechnologists certainly be worked on regardless of the prescreening outcome

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Performance Criteria

Wattenburg listed (due to Wied) performance criteria which all automated prescreening systems should meet. Among these is the requirement that any automated prescreening device be capable of processing 120 cases per hour or 1 case every 30 seconds. For machine prescreening in this allotted time interval, it is desirable to have a large number of cells present in the sample for good statistical accuracy. Husain points out that the ratio of abnormal-to-normal cells in a sample depends on where and how the sample was collected. This ratio can be as low as 2.5/100 in the vaginal sample or as high as 110/100 for samples taken by the endocervical cotton-type applicator. In addition, Wied has shown that variability in this ratio also depends on the degree of progress of the cancer. His studies showed that this ratio could be as low as 0.5/100 for subclinical early invasive or noninvasive cervical cancer (vaginal smear) to as high as 28/100 for clinically evident invasive cervical cancer (cervical scraping). Difficulty will arise when this ratio is low because of poor sampling, the state of the cancer, or a combination of both.

Speed of Automated Cytology

Under present circumstances there are two machine approaches under development: (a) computer-assisted single-cell scanning, and (b) low-resolution measurement of one or more cellular parameters with *flow systems*. Various best estimates of time necessary to scan a single cell have been offered, varying from 1 cell per second (Wattenburg) to 0.3 cell per second for one level of gray. Taking the faster rate, 30 cells would have to be scanned in a machine meeting the above-mentioned criterion of at most 30 seconds per sample. Using the figures above for the ratio of abnormal-to-normal cells, the range in number of abnormal cells expected in a sample of only 30 cells (assuming cells are classified as only normal or abnormal) would be 0.44 to 13.6 (Husain) or 0.15 to 6.6 (Wied); therefore state-of-the-art scanning systems are probably not capable of diagnosis for early stages of cervical cancer or in cases where the anticipated number of abnormal cells is low due to poor sampling. In other cases the total number of cells considered is low, leading to poor statistics on which to base a decision. If accurate and reliable decisions are to be achieved within the time specified by Wattenburg, it appears that scanning systems are not up to the task.

Flow systems can perform rapid analysis. Under present conditions, flow systems with optical sensors can measure 10^4 to 10 cells per minute, or one sample of 5000 cells well within a minute. With a scanning system operating at 2 seconds per cell for one level of gray, it would take 167 minutes to analyze one sample of 5000 cells. It is unlikely that one level of gray is sufficient information for making a diagnostic decision, thus increasing the time necessary to process a sample. Based on a 2000 hour working year and an average cytotechnician load of 5000 cases per year, present scanning devices are at least 7 times slower than the present manual method. The acceptable machine rate proposed by Wied is 50 times the current human rate.

Scanning systems appear to be largely limited by the time necessary to transport a cell on a slide (or moving tape in the Vickers method) under the microscope objective and to focus. With the Los Alamos Scientific Laboratory microfluorometer, cells move through the light beam at several meters per second, and the sampling time in the light beam is 30 μsec for one parameter. It is not difficult to reduce the sampling time by a factor of 5 to 10. Multiparameter analysis probably will not increase the time necessary for signal processing if a small computer (such as the PDP-type) is used. If small-angle light scattering and nuclear fluorescence were measured simultaneously on each cell, yielding cell size and DNA content, then one could store these two data, construct a nuclear-to-cytoplasmic ratio, and store it in about the same time necessary to process one parameter with a pulse-height analyzer. Thus, it is not likely that multiparameter analysis with flow systems will significantly reduce the speed of the method which, from the above arguments, is at least 200 times faster than scanning methods. Scanning systems have been under development for about 15 years, flow systems about 5 to 6 years. Increases in speed of electronics over the last 15 years have helped to decrease the analysis time of scanning systems, but the slowness of mechanical transport remains. This is not to say that a breakthrough cannot occur; however, further increases in electronics speed will give flow systems even a larger edge over scanning systems than at present.

Cost-Effectiveness Considerations

It was estimated in 1968 that there were 66 000 000 women 20 years or older and about 4500 cytotechnicians, each capable of handling 5000 cases a year for Papanicolaou prescreening. However, the manpower requirement is 13 200 (and pathologists) who provide systematic review of abnormal specimens by the more experienced and skilled.

It may be that the first and simplest task of automation in cytology screening should be at the lowest possible echelon of the hierachy i.e. separating perfectly normal specimens from all others But even this limited goal so far eludes us Only the most naive really believe that the cancer cell can be identified through a single parameter such as nuclear diameter and multiparameter measurements of cells have been technically complex, expensive and slow Basic guidelines for an effective multiple parameter system are outlined by Wattenburg and minimum performance standards by *Tolles*

The greatest effort has been expended in developing high resolution scanning instruments to make use of pattern recognition techniques in cell identification Rosenberg *et al* describe their own preliminary results with this approach but they do not offer the means for solving many technical problems encountered by earlier investigators who used the same general method

Novel optical techniques deserve more attention than they have had. The brief discussion of complex optical filters by Schmidt—Weinmar seems to offer intriguing possibilities for simple speedy classification or matching of cell types according to multiple photometric parameters Holography is still in its infancy but on theoretical grounds alone it demands more attention from us

A most promising method for carrying out multiparameter spectrophotometric cell measurements at very rapid rates has been offered by Kamentsky His proposal for examining cells in suspension as they flow through an incident beam of light solves many of the mechanical problems inherent in other systems of cell transport Hulett and Herzenberg indicate that there are already a number of specific applications for this technique of automated cell measurement, and his summary of recent work justifies speculation about a wide range of other applications in research and clinical laboratory medicine In addition to measurements of cell constituents, he points out the possibility of measuring cell function, as illustrated by a very nice study of antibody producing spleen cells Almost as an afterthought, Kamentsky suggests that the low resolution fluid transport system described by him may be capable of specific morphologic measures Hitherto, these could be obtained only with very slow high resolution devices and pattern recognition techniques, scanning a few selected individual cells on a quartz or glass slide

If morphology *and function related* parameters can be measured simultaneously with cell constituents at the ultrarapid rates of Kamentsky's fluid transport cell spectrophotometer then we do indeed have a powerful and exciting new tool for use in cytology

MR MEYER

Diamond has suggested a simple method of screening which will aid a technician in placing specimens into negative and atypical groups. He suggests an estimation of the eccentricity of nuclei by measuring their maximum and minimum diameters, and has found, on average, that the minimum nuclear diameter is greater than 12μ in atypical smears. I agree with him that one parameter is sufficient for preliminary screening, *because there is a lack of a common general* classification.

Shuster *et al* described various ways in which cervical material may be analyzed automatically As these authors state the Papanicolaou staining method gives a vast slice of information. However the interpretation of atypical specimens varies from laboratory to laboratory and I believe that a common agreement in coding is now essential.

A new aproach to the handling of the specimen prior to analysis is described by Heanley The advantage of this method is that the cells are separated and then randomly associated, a positive step in the presentation of material for both manual and machine analysis, as any unit area of the sample is then equivalent in terms of material to any other unit area.

Tolles reiterates my earlier comment that the level of performance varies from one demographic and clinical situation to another He implies, I believe that a tool which will increase the technician's output —without any compromise in diagnostic capability —will be acceptable I would add that any machine must not restrict the individual laboratory in its own definition of negative and atypical material

Husain clearly points out the main problem in multiple parameter analysis—the problem is one of resolution versus time

In conclusion, I would say that until quantitative descriptors replace the present commonly approved qualitative descriptors there is no possibility of producing an automatic analysis system Until that date all that can be done is to produce a mechanical system which will increase the productivity of the cytotechnician The designer of complex systems has no specifications with which to work until the cytotechnologist can quantitatively express the parameters of analysis

DR. MULLANEY

The contributors of opening remarks on this topic have dealt mainly with single-cell scanning devices with the exception of Hulett and Herzenmated systems. Hence one's judgment would fail on machine-human interaction of the Vickers Cytology Screening apparatus.

Finally, the application of specimens in the Vickers system is full of unsolved difficulties. Based on several trial runs with this system, and with careful rescreening, usually the biggest problem always was that the cells on which diagnoses could be made were peeling from the tape. This occurred in spite of an adequate overall cell content of the specimen. Our preliminary runs of close to 5000 samples indicated successful sample preparation in about 20 per cent of those which should have contained malignant cells. Modification of chemical processing in our laboratory enabled us to improve this to over 75 per cent. However, to this date the sample preparation problem has not been resolved in [illegible] satisfactory way, and no satisfactory warning mechanism exists for discovery of this type of malfunction.

Re Dr Hendry Opening Remarks

Regarding the contrasting advantages and disadvantages of man and machine, one important consideration was omitted. Machines can be divided and recombined to suit the operator. Thus, entirely different parameters can be evaluated in [illegible] *random* way if one chooses. It is very difficult to reprogram man completely. Past errors as well as falsely interpreted instructions linger for an unspecified time. Hence troubleshooting [illegible] machine is also [illegible] far easier task than the analogous process with man.

Re Dr Husain Opening Remarks

Dr Husain has given the most beautiful summary of this field and we should all be grateful to him. On one point I should like some clarification.

Dr Husain mentioned hyaluronidase as [illegible] cell dispersing agent. This suggestion has been in the literature for some time but I have not been able to find encouraging reports of experimental data. From [illegible] biochemical point of view hyaluronidase should not have [illegible] lytic effect on cervical mucus. However, it is possible that relatively impure enzyme in the order of 300 to 500 T.R.U. per milligram will contain sufficient activity of proteolytic, or other enzymes to accomplish the desired lysis. Since highly purified hyaluronidase is available the point can be resolved.

From [illegible] biologic point of view some enzyme for testicular extract must have this activity since sperm must be transported through the cervical mucus in order to accomplish their allotted task. Hence it may be possible to isolate an enzyme with specific activity.

Re Dr Kamentsky Opening Remark

[illegible] sample preparation problem has not been solved. The problem applies equally to rapidly flowing and substrate deposited cells.

Little experience with *tape transport* of cells. This really means little published experience. In practice variations in focus are not a problem, as can be determined by the sharpness and reproducibility of waveforms while the tape is in motion. Cell overlap can be [illegible] serious problem and its resolution was [illegible] effort of chemistry preparation of samples. Background irregularities are no greater problem here than in any cytologic automation.

The use of the word spectrophotometer associated with automated cell analysis has puzzled one for [illegible] long time (see Science 150: 630 (1965)). The word or phrases incorporating it imply that spectra are taken and analyzed continuously while cells are being scanned. Having read and re-read these papers it seems evident that this just is not so. Isolation of colors by filters leads to photometry whether for [illegible] bulk or microscopic object. Since there are techniques for true rapid scan microspectrophotometry, one may suggest that the use of phrases implying spectral analysis be discontinued where these techniques are not under discussion. The above comment in no way reduces the importance of [illegible] resolution processing of cells and the significant gain [illegible] speed provided thereby.

Re Dr Wettenburg Opening Remark

When multiple parameters are each used singly in [illegible] multicycle program, far less information is gained than by using multiple parameters on [illegible] single pass. The analogy which is easiest to understand in this regard is that of [illegible] man counting black and white dogs of both sexes passing through two gates. If at one gate he records color and at the other gate sex, he will never know how many white bitches have passed the gates. If he records both at one gate he will have information on four separate populations. Since there are always many populations from which to identify a variety of diagnoses, the serial parallel design described by Dr Wettenburg is dangerous and misleading.

There are ten papers offered on the subject of approaches [illegible] prescreening.

Kamentsky and Hulett and Herzenberg are concerned with cell suspension flow systems. Kamentsky reasons that opto-electronic machines mimicking cytotechnicians with serial scanning would be impossibly slow and expensive. He describes [illegible] complete system claiming relative simplicity while making four simultaneous measurements on each cell. Hulett and Herzenberg give [illegible] more general review of flow systems mentioning technicians if it is desirable to screen the entire female population over 20 years of age. With the 1968 rate of training 500 cytotechnicians a year by 1985 sufficient numbers will be available to meet the 1968 requirement, assuming no attrition. Clearly it is impossible to rely on the present human method to screen all women for cancer of the cervix unless more technicians are trained. Some type of automation is necessary. If a flow system prescreening machine could handle a case every 5 minutes (1-minute measurement, 4 minutes down time) and run full time (24 hours per day, 365 days per year), 630 machines would be required to screen all 66 000 000 adult females in the United States. Estimating costs at $50 000 per machine, $5,000 per year upkeep, and $15 000 per year for technician/operator, and 3 technicians per machine, the total cost of such a screening operation over the 5-year period before a new generation machine might evolve would be $189 000 000 (assuming no inflation). At $15 000 per technician, the total cost of human prescreening by 13 200 technicians during the same period would be $990 000 000 (i e, the automated method costs only 19 per cent of the projected human approach). This assumes, of course, 100 per cent accuracy on the part of both methods; clearly this is impossible. The cost of machines in the automated method is only 22 per cent of the total. Thus, a larger number of machines could be purchased to handle the recalls, and still the cost-effectiveness of the automated method could exceed that of the present method.

These numbers could be argued, but the point is that, even at worst, an automated flow system would not be more expensive than the human approach and would be able to reach the entire female population, which is not possible under the present conditions. Obviously, lack of speed and high initial cost of present scanning systems leave them out of the competition for prescreening at the mass level. Flow-system sensors can be coupled with separators to isolate the abnormal cells for a second prescreening step by a scanning machine looking at each cell in more detail. As Wied has suggested, the ideal automated approach to screening would consist of a flow-system prescreener coupled to a separator and a scanning system for analysis of suspect cells in more detail. The output of these machines would then be given to a human observer for final decision. Flow system sensors exist which can measure light scattering, fluorescence, etc. However correlations have yet to be made between the parameters these machines can sense and the biologic state of cells. It is in this area that considerably more research effort is necessary.

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DR SAUNDERS

Re Dr Diamond's Opening Remarks

In reference to the device which screens cervical cells on continuous reels of tape, there are several points which must be made.

First, it is not a completely automated device where one puts in a raw specimen and has an output of yes or no for cervical dysplasia and cancer. This lack of complete automation can be good or bad, depending on one's point of view. Intelligent interaction of human with machine certainly can provide a powerful methodology. Provided the human can keep up, productivity will increase at least by an order of magnitude. Second, there must be some very carefully designed safeguards built into such a semi-automated prescreening device. These include an assurance of an adequate specimen in terms of both quantity and quality. Presently this does not exist. The closest approximation is the presence of grit and dirt on the tape referred to by Diamond. This will trip the marking device and force the operator to look at the specimen.

Thirdly, during a cursory view of the specimen, such as at a false positive mark, ancillary diagnoses such as inflammation, trichomoniasis infestation, and bleeding could be made. These ancillary conditions are usually represented by cells or material generally distributed on the slide or tape. The ability to make ancillary diagnoses of great variety is not yet feasible in completely automated systems Hence ones judgment would favor machine-human interaction of the Vickers Cytology Screening apparatus

Finally the application of specimens in the Vickers system is full of unsolved difficulties Based on several trial runs with this system, and with careful rescreening finally the biggest problem by way was that the cells on which diagnoses could be made were missing from the tape This occurred in spite of an adequate overall cell density of the specimen Our preliminary runs of close to 5000 samples indicated successful sample preparation in about 20 per cent of those which should have contained malignant cells Modification of chemical processing in our laboratory enabled us to improve this to over 75 per cent However to this date the sample preparation problem has not been resolved in satisfactory way and no satisfactory warning mechanism exists for discovery of this type of malfunction

Re Dr Hawkins Opening Remarks

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Re Dr Wattenburg Opening Remarks

When multiple parameters are each used singly in multicycle program, far less information is gained than by using multiple parameters on single pass The analogy which is easiest to understand in this regard is that of man counting black and white dogs of both sexes passing through two gates If at one gate he records color and at the other gate sex, he will never know how many white bitches have passed the gates If he records both at one gate he will have information on fou separate populations Since there are always many populations from which to identify variety of diagnoses, the serial-parallel design described by Dr Wattenburg is dangerous and misleading

There are ten papers offered on the subject of approaches to prescreening

Kamentsky and Hulett and Herzenberg are concerned with cell suspension flow systems Kamentsky reasons that opto-electronic machines mimicking cytotechnicians with serial scanning would be impossibly slow and expensive. H describes complete system claiming relative simplicity while making four simultaneous measurements on each cell Hulett and Herzenberg give more general review of flow systems mentioning especially the use of fluorescence and the desirability of measuring two or more parameters

Rosenberg describes a study of possible parameters using photographs and concludes that unique descriptions of single cells will require a considerable number of *measurements. He offers* no complete system. Diamond put forward the hypothesis that the use of a cut-off point comprising a diameter of 12 *microns will distinguish normal* from suspicious cells in cervical cytology samples.

Shuster *et al* lists and discusses various single *parameters but concludes that any single* one of them by itself is very unlikely to be helpful Husain like Shuster *et al* discusses various parameters and method

Tolles sees prescreening as an incomplete and possibly inefficient step due to the resultant in*crease in work load and fall in throughput* Heanley also questions *the rationality of* prescreening, before listing the advantages of track *transport for cellular specimens*

Schmidt Weinmar outlines a theoretical method based on optical diffraction This is a comparator *method and his complex filters would be designable* only after a full description of the object to be detected is available Wattenburg in his review states some of the *practical requirements for* automated prescreening systems He favors a multiparameter approach of sufficient efficiency to give the automated device as good as or better results than cytotechnicians His final comment on the need for "clean" specimens points out the need for integration between preparation and sensing

None of the papers carry a strong argument for prescreening, in fact, several imply that prescreening will create more problems than it solves Several authors would like to see a final human decision but are uncertain how to reconcile this with the difficulty in matching machines to people and the fact that most decisions will irrevocably be made by the machine Another underlying theme is that multiparameter systems are required only Diamond opposing such a conclusion.

The cell suspension flow systems have a more unified appearance presumably because they are altogether new and the preparation and sensing are physically much more intermingled than systems using solid substrates for cell

It is therefore thought that automatic screening must be approached on an all or nothing basis and the present method using cytotechnicians and slides can only be *reasonably* replaced by a true multiparameter analyzing system constructed with due regard to the stochastic nature of the processes involved. This *implies that prescreening is not* likely to be of economical use in routine applications and the attempt to match machines and cytotechnicians in a single system is misleading

Dr. Ward

The earlier work of Diamond[2-4] *and Rimmer*[1] appeared to demonstrate satisfactorily that positive cervical smears have a higher frequency of cervical cells with large nuclei compared with negative cervical smears. The further work of Diamond, reported in his "Opening Remarks" confirms this conclusion

It remains to construct a machine which is capable of distinguishing cervical cells with large nuclei from other confusing objects in the sample such as small clumps of white blood cells.

The development of such a machine depends on several related factors

1) Improved preparation of the sample to reduce the frequency of cell clumps (mucus-reducing agents—ultrasonic)[6]
2) Improved presentation of the sample to aid machine analysis (film strip)
3) Production of a genuinely practical economic scanning system with adequate signal to noise resolution and stability at the scanning speeds required (television or mechanical scan)[7-]
4) Evolution of a recognition strategy which has been proved capable of distinguishing large cervical cell nuclei from other confusing objects (See McMaster this Symposium)
5) Production of computing facilities to implement economically *the recognition strategy* (small computer)

All of these factors are being examined by independent research groups in the United Kingdom with integration and cooperation encouraged by meeting together as an informal group

Steady progress is being made and there does not appear to be any insoluble problem

It seems to be generally agreed that a machine should be taken carefully and sensibly through a prototype stage to optimize parameters and, of course such a machine only has to carry out its task as cheaply and quickly and no worse than the average trained human observer It is difficult to estimate how bad the last is, but one would not be surprised to learn that the average number of positive smears found in the same collection of cervical samples by trained human observers in *different clinics using the conventional cytologic* classification system varies by as much as 10 to 20 per cent. This is probably due partly to observer *errors and variations*, but is also due to intrinsic difficulties in defining precisely the morphologic characteristics of tumor cells

It may well be that the Diamond criterion is in fact superior to the conventional system of classification It certainly seems no worse and is precisely defined.

It seems likely that the ultimate practical machine for cervical screening will be the same, with minor modifications, to that required for white blood cell differential counts and possibly number of other related routine diagnostic procedures involving microscopy

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DRS WHEELESS AND PATTEN

A prescreening device for automated cytology should be capable of screening out all obviously normal samples, leaving only abnormal and questionable samples for the cytotechnologist and cytopathologist It must do this with false negative rate equal to or less than present manual techniques and with false positive rate sufficiently low to justify its existence Any sample judged not normal by prescreening device must be returned unharmed to the cytologist for examination by conventional morphological techniques In addition, because there are approximately one-hundred thousand cells on typical cytosmear the instrument must be capable of examining cells at rate approaching 500 cells second to prescreen single sample in an acceptable time period

Abnormal cells manifest themselves in an extremely large somewhat undefined region of multi dimensional cellular parameter space At present it is difficult, if not impossible to precisely define the boundaries in this space for the various sub-sets of abnormality It would seem better to concentrate on recognizing normal cells (and known non-cellular artefacts) because they fall within more limited and well defined region in

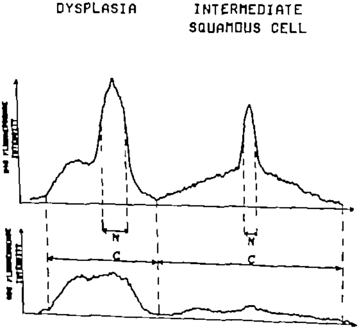

FIG 10. Slit-scan contours recorded at 540 nm and 655 nm on dysplastic and an intermediate squamous cell.

this multidimensional cellular parameter space. A prescreening instrument must make a decision on each cell in real time and if it lies outside the region for normal cells, then that entire cellular sample must be retuned to the cytotechnologist.

In a previous article the advantages and disadvantages of zero and high resolution optical systems were discussed. It was emphasized that a low resolution slit-scan system offers certain advantages over other systems for prescreening. It represents a compromise solution, a tradeoff of resolution and throughput. On this basis, the present approach to prescreening has been to develop a multiwavelength, slitscan fluorescence system.

A fluorescence method was chosen because it avoids many of the problems associated with micro-absorption techniques and because it provides increased sensitivity. While a final system would be a flow-through system to achieve a desirable cell throughput rate, an initial instrument records slit-scan contours from cells on slides. This instrument allows one to relate recorded and calculated parameters to a cellular classification based upon morphology.[1]

The slit-scan concept involves recording the secondary fluorescence of a cell at discrete time intervals as the cell passes under a slit aperture. The cells are stained with the metachromatic fluorochrome acridine orange. Secondary fluorescence slit-scan contours are recorded at 540 nm and at 655 nm. Secondary fluorescence at 540 nm is reportedly related to the DNA content of the cell while cytoplasmic and nuclear RNA reportedly exhibit red secondary fluorescence (655 nm). Non-specific (non-DNA related) green fluorescence, measured at 540 nm, was observed to occur however in the cytoplasm of mature squamous cells. This non-specific cytoplasmic staining in squamous cells was observed using any of the current fluorochrome staining techniques for DNA, such as those developed by Ruch, Rigler[3] and Von Bertalanffy, Masin and Masin.[4] Thus non-specific, green cytoplasmic fluorescence unless recognized as such, will mask the expected difference in fluorescence between a normal cell and an abnormal cell having an increased DNA content in the nucleus but lacking any appreciable cytoplasm.

The slit-scan contours recorded at 540 nm and 655 nm on a dysplastic and an intermediate squamous cell are illustrated in Figure 10. From

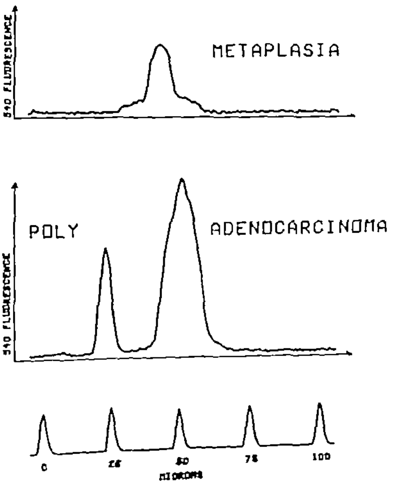

FIG. 11. Slit-scan contours recorded at 540 nm on cells derived from the human uterine cervix. The scale the bottom of the figure resulted from the scan of precision ruled scale.

such contours the fluorescence from the nucleus of each cell can easily be distinguished from nonspecific fluorescence in the cytoplasm. The computer integrates the area under the nuclear portion of the curve to obtain a measure of the DNA content of the cell.

Of equal importance is an additional parameter gained with this technique. Since the boundaries of the nucleus and cytoplasm are recognizable from the fluorescence contour, the nuclear cytoplasmic ratio is also available for use in cell classification. From the 655 nm fluorescence contour in Figure 10, an increased RNA content in the dysplastic cell is evident.

It should be noted that for the 540 nm fluorescence contour, the areas under the curves for the two cells are approximately equal due to nonspecific fluorescence in the cytoplasm of the normal intermediate squamous cell and the increased fluorescence in the nucleus of the dysplastic cell. However, the fluorescence from the nucleus of the dysplastic cell is approximately twice that of the nucleus of the normal squamous cell. Thus recognition and isolation of nuclear fluorescence by use of the slit-scan contour permits a classification decision to be made that could not otherwise be made by comparing total 540 nm cellular fluorescence alone. The classification decision, based on an increase in nuclear fluorescence, is reinforced by an increased N/C (nuclear cytoplasmic) ratio for the dysplastic cell and an increase in 655 nm cytoplasmic fluorescence.

Figure 11 illustrates some 540 nm fluorescence slit-scan contours recorded on other cells derived from the human uterine cervix.

The upper curve represents the fluorescence scan contour obtained on a cell derived from squamous metaplasia, a common benign surface reaction in the cervix characterized by a smaller squamous cell size with an increased N/C ratio. Note that while the N/C ratio is elevated, there is no appreciable increase in nuclear fluorescence.

The middle curve depicts a fluorescence scan contour across a polymorphonuclear leukocy

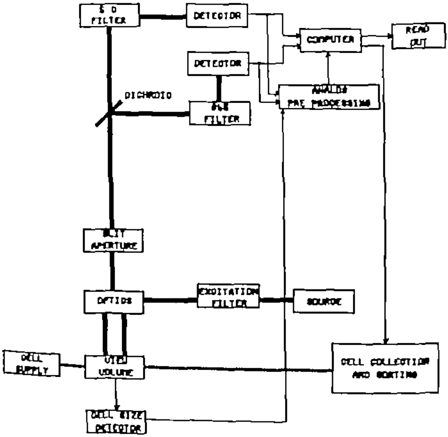

Fig. 12. Block diagram of flow-through slit-scan prescreening system.

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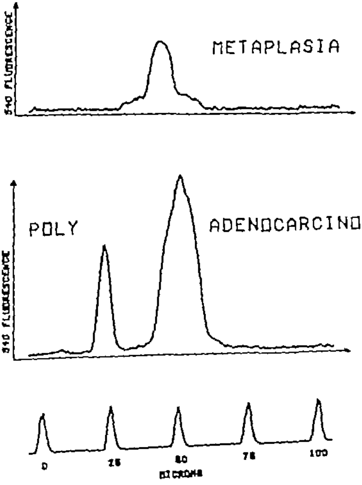

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better than slides This is true for speed and ease of handling, but slides should not be ignored as the cell sample can be immediately read by orthodox microscopy Moreover if a code signal system is stamped on the "label" area of the slide—or on a slide carrier card—to register the presence of abnormal nuclei in various parts of the slide it only requires a simple automatic signal-finder attachment to the average microscope to permit any slide automatically prescreened, to be check-screened by cytotechnologists on their own microscopes

I think it is very necessary to identify the cost effectiveness of the various procedures as Dr Mullaney has done but Dr Holm makes the plea to look at sampling I would say this is the most expensive aspect of the system, and serious thought should be given to better and easier forms of collection The irrigation sample is by no means as good as the scrape smear but has the cytopipette been developed enough. We ourselves have found it 80 to 90 per cent as accurate as the scrape smear and the cost of collection only about one-eighth to one-tenth that of collecting the scrape smear

I agree with Wied about two or even three stage scanning system, but surely a flow system transferring to a scan system is not as easy to create and run as a double scan system the first a fast seeker and the second a slower higher resolution analysis system The whole can be in series on the one form of presentation which is permanent

Dr Bibbo asks about signal-to-noise ratio of the Quantimet I believe I am guilty of misrepresentation in terms here I quote the signal-to-noise ratio as one where the "noise" is cytoplasmic and not just background It is a useful term in this regard as it is the one measurement which identifies whether or not the scanner can read nuclear detail well above the surrounding "densities" on a monochromatic scan of light transmission This, of course varies with the type of stain used, the color filter used, the cell presentation with cytoplasm overlaps and clusters causing dense cytoplasmic light absorption, and the range of nuclear staining intensity of the malignant, non-malignant, and degenerate nuclei

Since the Quantimet potentiometer for light absorption is only roughly linear and not directly translatable to absorption coefficients or light transmission, I can only give you the arbitrary figures recorded on the potentiometer dial From nil to 100 per cent light absorption, there are 400 arbitrary units, and with the average Papanicolaou stain using tungsten light, the difference between malignant nuclei and cytoplasmic background is only 60 to 80 units at best. Of course, with other stains, filters and improved cell presentation we have improved on this, but I think we still have a long way to go

Dr Saunders asks a question regarding hyaluronidase Our experience with the agent has been small and mostly in connection with the alteration of viscosity of alimentary and respiratory mucus secretions Our work was admittedly with the impure enzyme but the point I wish to make in regard to all methods of cell cluster disruption and cell dispersal is this For many years little work was done on this vital aspect of automated cell analysis now everyone is concerned, and the aim is to achieve a uniform, homogeneous disrupted and dispersed gruel like spread on slide or tape The very fact of doing all this could remove 50 to 70 per cent of the other criteria of malignancy or suspicion thereof The cluster is torn apart, the cell debris and cancer diathesis disappears The fluffy degenerate adenocarcinoma cell is destroyed completely and all these are the cardinal signs of malignancy sought by the cytotechnologist when confirming the malignant sample With our Quantimet and Cytoscreen apparatus and the Vickers machine the principle of signaling abnormal cells or fields for confirmation by eye demands preservation of as many of these criteria as possible

Surely a compromise is needed here By all means separate some cells but keep them in good state Enzymes of the trypsin type can do much damage Physical methods such as atomizing or even ultrasonics tend to damage less The aim with Cytoscreen is to use the electronic circuitry to cancel the false positive and try to define the true positive signal One dispersed malignant nucleus will act as a signal and the microscope field may still have the cluster from which it derived, and it is this that makes the diagnosis for the cytologist

Perhaps I will be forgiven if I record the apt comment—of which I was not the author Why seek further for a computer to make the final diagnosis The best computer is the human brain—and it has the virtue of being mass-produced by unskilled labor

DR KAMENTSKY

I am pleased that Dr Cram, Dr Ehrlich, Dr Gallus, Dr Holm, Dr Melamed and Dr Mullaney in their Discussions have placed their bets for the near term as I have on multiple parameter flow systems for prescreening I would like to state, however that pattern recognition systems, because of the greater amount of information extracted from the cell and processed, will potentially provide finer distinctions among cell types than will zero resolution systems Although it is slow and expensive now high resolution scanning should be considered as a second stage to a flow system prescreener or as Dr Wied and his collaborators have done so well, to assist the cytopathologist with problem cells Pattern recognition and a cell derived from adenocarcinoma Note the considerable increase in nuclear 540 nm fluorescence in the abnormal cell The present investigation requires recording and analysis of fluorescence contours at 540 and 655 nm from as many normal and abnormal cell types from the human uterine cervix as possible to establish a data base These measured parameters are being correlated with visual cell classifications based upon morphology [1]

Figure 12 illustrates the next step in this program which is the automation of the slit-scan concept into a flow through system to obtain the required throughput of several hundred cells per second Cells from the cell supply and staining station will flow in solution through the view volume where their fluorescence contours at 540 and 655 nm will be recorded From the contours the computer will make a decision on each cell in real time

The detection of an abnormal cell will tag that particular sample for reexamination by a cytotechnologist or cytopathologist The particular cells judged abnormal by the computer could be physically separated from the rest by incorporating a fluidic gate under computer control into the cell collection unit

In conclusion, results to date indicate that an automatic prescreening instrument for diagnostic cytology based upon secondary fluorescence contours of individual cells obtained by a slit-scan technique, is both practical and feasible

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Closing Remarks

DR. HEANLEY

On careful reading of the discussions on approaches to prescreening I find it impossible to really draw any conclusions, except that no one seems to have either a coherent complete theory or any practical device at the present time and most of the comments are purely repetitive

DRS. HULETT AND HERZENBERG

There is very little that we wish to add at this time. We are in general agreement with the comments on our contribution and also with the repeated idea that flow systems possess inherent advantages over scanning systems where large numbers of cells must be observed.

DR. HUSAIN

Dr Mullaney has hit on some vital and controversial points. His rate of scan requirement is a bit high, I think. If 3 minutes to scan 50 000 cells is acceptable, and a track scanner such as the newer version of the Quantimet image analysing computer Cytoscreen (now in our hands for trials) should achieve this, then the limited target of marking the field containing an abnormal malignant cell should permit a technician to screen well over 50 000 cases per year or achieve more than 7 to 10 times the normal rate Any instrument costing £5 000 to £10 000 with depreciation and repair costs of 10 per cent, equates to the salary of one technician a year The benefits are thus 7 to 10-fold.

I do not agree entirely with Dr Holm in his assessment of priorities and the preference for the multiparameter approach *I think* we are in ultimate need of the latter but as an interim solution the simplest and fastest way of alerting by eye to view the fewest possible fields in a specimen with a concern to reliability we are bedevilled with the matter of resolution versus speed and to get "within the nucleus we would have to get down to a resolution of picture point of 0 25 μ as happens with the Cytoscan The Cytoscreen and Quantimet on a ×20 or ×25 objective magnification, can reach down to a 0.7 to 1 0μ only With these limitations we have utilized the single spot scanner with line memory and logic circuitry to its maximum in choice of exclusion criteria and we hope the elimination of the false positive material will be maximal while still registering the true signal The problem we face is the inability to register malignant nuclei below the diameter of 10 or 12μ as leukocytes and inflammatory superficial squame nuclei cause interference

I agree wholeheartedly that flow systems have the benefit of speed, but our assumption has always been that an automatic prescreening device would be satisfactory if it matches the speed of a technician screening an "easy" or "clean" smear Moreover the tape or slide track presentation does permit immediate viewing and checking by eye.

It is further mentioned that film track would be better than slides This is true for speed and ease of handling, but slides should not be ignored as the cell sample can be immediately read by orthodox microscopy Moreover if a code signal system is stamped on the "label" area of the slide—or on a slide carrier card—to register the presence of abnormal nuclei in various parts of the slide it only requires a simple automatic signal-finder attachment to the average microscope to permit any slide automatically prescreened, to be check-screened by cytotechnologists on their own microscopes

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I would like to answer A M Saunders specific criticisms of my previous publications and Opening Remarks. I thank him for bringing to my attention for the first time since *Science 150* 630 1965" that the word "spectrophotometer" is probably not used appropriately for a multiple wavelength zero resolution flow system. I will be more careful of its use in the future

In our publications on rapid cell measurement we have always tried to make the point that preparation problems (sampling, isolating, fixing, and staining cells) in suspension are different than those of cells deposited on a substrate such as a slide or tape. For some applications they have been solved for liquid flow systems and the applicable techniques have been described For the prescreening of cervical cancer this was perhaps our major problem and this was not satisfactorily solved I agree with Dr Saunders that all sample preparation problems have not been solved.

I know of no published solution to the problem of routinely scanning cells deposited on a tape with sufficient resolution (0 5 μ) to obtain the detail

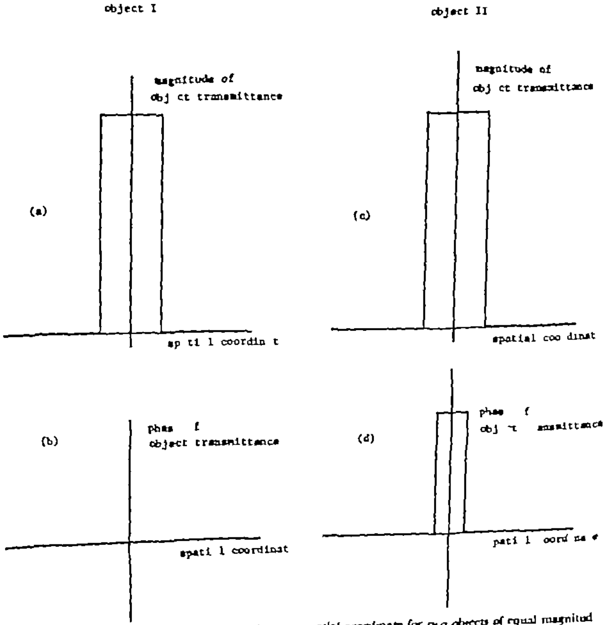

Fig 13 Transmittance (magnitude and phase) versus spatial coordinate for two objects of equal magnitude but different phase (~refractive index) of transmittance function

required to characterize the morphologic distinctions normally needed by cytotechnologist. To simply scan across the cell to measure diameter and to observe the waveforms produced is much mpler problem. Background irregularities due to stain and substrat will be superimposed on the signals derived from cells i any zero or high resolution scanning system I nattended rapid

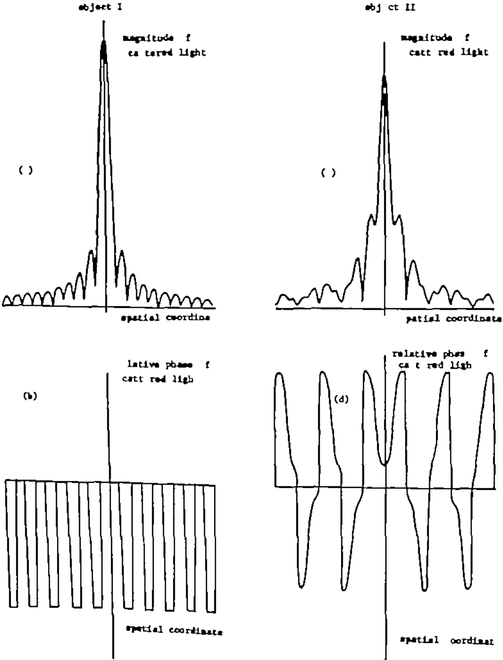

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Fig 14 Magnitude (~sqrt intensity) and relativ phase of light scattered by the two objects given in Fig 1

processing systems we found the homogeneity of solutions in which the cells are suspended provided a distinct advantage in eliminating this problem. I must therefore disagree with Dr Saunders on these points.

DRS. ROSENBERG AND KLINE MESSRS. LEDLEY AND MARKOVITZ

We have reviewed the discussions you sent us and have decided not to submit closing comments.

DR. SCHMIDT WEINMAR

This discussion seems to show that at the present time the most promising concept of an effective and economic prescreening technique points to a flow system in combination with instruments to rapidly measure more than one parameter Relevant to this, I would like to mention the qualifications of complex optical filters it seems quite possible to identify small semi-transparent particles of both given refractive index and given geometry by using a one-step complex optical filtering system.[1]

Consider for instance, the case of a particle of given size and of two different given values of the refractive index (Fig 13) If the refractive index varies, the intensity of the diffracted light as measured in the far field does not change much in this case but the relative phases of the scattered waves do show considerable change (Fig 14) Using known principles (refer to the reference list following the "Opening Remarks" this chapter) one can design optical filters which differentiate light according to both its magnitude and phase distribution Such a filter may for instance strongly respond to magnitude and phase of the scattered light as given in Figure 14 (a) and 14 (b) while it removes light of the distributions as given in Figure 14 (c) and 14 (d) Hence, it discriminates between particles according to their size and refractive index The strength of the response of this filter can be used for generating a signal every time a particle of given size and refractive index passes the measuring spot of the flow system.

Though the examples shown here are extremely simple they demonstrate the qualification of complex filters to identify semi-transparent objects by simultaneously detecting more than one descriptive parameter

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DR. SHUTTER

The discussions on "Approaches to Prescreening have emphasized the problems—some of them basic—which have retarded the development of an adequate prescreening method by machine.

The most significant of these problems is the lack of exact quantitative criteria for defining malignant or premalignant cells. Many of the criteria used in current prescreening attempts are based on visible properties but serious consideration must be given to the development of criteria based on cell properties not visible or not presently obvious which will be more amenable to machine detection. Until these criteria are established, no screening system can be perfected

The methods of screening currently being attempted fall primarily into two categories 1) image processing and 2) analysis of a suspension of cells Image processing is expensive time consuming and rigid in its requirements for specimen preparation, but it is potentially capable of distinct diagnoses. Th analysis of a cellular suspension should be less expensive faster and more amenable to a multiparameter approach. Most investigators have stressed the need of desirability for some pre-processing to eliminate artefacts and concentrate the population of cells to be screened An added task of significance is the collection of the cells for subsequent repeat analysis or visual screening A satisfactory specimen can be obtained without the need for a physician but the absence of a complete medical examination is a drawback. Also if no slide is produced of the cell studied, the detection of other non-cancerous abnormalities would be limited

DR TOLLES

The three keys to effecti e automatic prescreening are adequate methods of cell collection to insure a high degree of cancer detection good preparative methods to ensure retention and emphasis on the abnormal cell complement and to reduce non-contributing background and finally the acquisition of highly specific and efficient criteria of malignancy which will ield high accuracy of classification It is approaching the academic to be too insistent on "standards when indeed we are still searching for more ffecti e quantitati criteria of suspicion Until that time it is entirely possible that g ing added attention to preparati e methods in combination with the method of cell collection would result in immediate gains in accuracy and productivity of the screening groups

DR. WATTENBURG

Dr Saunders comments on this paper are not the most appropriate criticisms which could be made if one reads the words as well as looks at the pictures. The text of the paper emphasizes design approaches to optimize enrichment through multicycle selection and to minimize mixed-positives through rejection of normals rather than selection of abnormals. For any given parameter or set of parameters being measured there is quite a difference between the threshold values for selection of normals with high probability and the threshold values for selection of abnormals with high probability. The little black squares in Figures 6 and 7 do not necessarily depict a single physically measurable parameter but rather the most complete analysis which can be performed at one point and time at the desired flow rates. For instance, the parameter P1 in Figure 6 would likely be nuclear-to-cytoplasmic ratio which is normally obtained by the measurement of two different physical characteristics, cell size and DNA content. Both of these physical characteristics can be measured at compatible rates and with similar types of electronic equipment without seriously encumbering the flow rate. However, optical scanning for morphologic characteristics could not be accomplished simultaneously with the above measurements without seriously reducing the flow rate attainable. These kinds of considerations are understood by those who have built systems such as the ones proposed in this paper. The use of the term "parameter" carries the implied interpretation given above. If necessary, an example such as the nuclear-to-cytoplasmic ratio measurement should be included in the paper to clarify the use of the word "parameter." However, it is still the case that this subject is not the main purpose of the paper.

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Cell Recognition by Multivariate Gray Value Analysis in Digitized Images

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Objective assessment of cell images[7] can lead to classification decisions which assign unknown cells to a given cell type with high likelihoods. Cytodiagnosis in the TICAS master program[11] follows computer processing of digitized cell images. Self learning programs extract a number of image properties from images of cells of known classification and establish, in a supervised learning process, a classification algorithm on the basis of information derived from such training sets.

Cell image properties selected for their discriminatory potential are mostly related to image texture[11]. Among these measures is the distribution of gray values as found in images belonging to cells of different types. The optical scanner is capable of distinguishing 180 different gray levels. It has been shown earlier however[8, 11, 13, 14] that 18 gray levels are not only adequate for most cell discrimination problems, but that grouping of gray values into intervals of 0.10 extinction units width counteracts cell to cell variability[8] and leads, in fact, to more consistent discrimination.

There are instances, however, in which it is advantageous to make use of the originally measured gray values and analyze them directly. This is the case when cells of different type or physiologic state show changes in gray value distribution which are small compared to a 0.10 extinction interval, because such discriminating information would be suppressed. This situation was encountered in gray value distributions[11] in the cytoplasm of virus infected Hela cells.[4, 6]

Transition from 18 gray level type analysis to 180 level distinction if required by the properties of the cell studied is easy since, of course, the primary data acquisition from the cells image is initially made from 180 gray levels. By studying mutual dependencies among the originally recorded gray values one should be able to arrive at a decision as to whether a cell came from one cell type or another assuming that the typical gray value dependencies for both cell types were well known.

It is the purpose of this paper to describe a computer program designed to assess the multivariate distribution of gray values in cells of different types, and to present results obtained by applying the program to clinical cell material.

MATERIAL AND METHODS

The clinical material consisted of 100 lymphocytes each from a normal human donor and from a case of asymptomatic chronic lymphocytic leukemia. The material was stained with Meyer Bennet hematoxylin. Preparation of the material, and analysis by the TICAS master program's supervised, and non-supervised learning sections have been described earlier.

A second set of cells consisted of lymphocytes from a case of benign lymphadenitis, and from a case of malignant lymphoma. This material was stained by Harris hematoxylin. The results of

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processing by the TICAS master program will be reported separately.[12]

All cells were measured on the Cytoscan microscope, at a wavelength of 570 nm, and with measuring spot size of 0.5 × 0.5 μ^2. Every cell image was cleaned by means of the TICAS 8 program.[13] From the recorded data sequence of seven consecutive measurements in a scan line through the center of each cell were selected for analysis of gray value dependencies. This corresponds to scan length of 3.5 μ in the object.

The program organization† and design rationale are briefly described under results. simple introduction to the underlying multivariate statistics is given in an appendix.

Program versions in FORTRAN IV are available upon request for the CDC 6400. Typical run time for training sets of 100 cells each is 8 secs on the CDC 6400.

RESULTS

Program Functions

The MVA (multivariate analysis) program reads sequence of gray values from preselected area in the cell image. A set of such sequences from two training sets each are used as samples of the underlying stochastic processes. A model of both processes made by estimating the means and covariance matrix in each case. The probability‡ that given sequence of gray values can be generated by each specific model can now be evaluated. Rather than find the inverses of the covariance matrices, the gray value variables are transformed to independent variables by finding the eigenvectors of each covariance matrix. Since the transformed variables are independent, the likelihood ratio can be readily computed and classification made for a sequence of gray values from an unknown cell.

In the present study processing of all cell images by the MVA program as preceded by subroutines finding the center of the cell, and selecting sequence of gray values along diameter through the center.

Applications

The two sets of lymphocytes from normal human donor (LNRM) and from case of asymptomatic chronic lymphocytic leukemia (ACLL) were recognized with an acceptably low error rate as shown by their classification matrix.

	LNRM	ACLL
LNRM	92%	8%
ACLL	5%	95%

A gray value sequence of seven values was used. The average likelihood of assignment to the normal, or the leukemic cell type was

	LNRM	ACLL
LR	5.77 × 10[illegible]:1	3.53 × 10[illegible]:1

The classification likelihoods attained by full data extraction of the TICAS master program are higher. However, only seven values per cell are observed here, and the assignment is made on the bases of the multivariate gray value distribution exclusively. A program option, to normalize for staining intensity by dividing through the average gray value of each cell's value sequence was employed. The recognition is thus based exclusively on relative value changes, or granularity. It is largely independent of staining intensity.

On data from benign lymphadenitis (LA) and from case of malignant lymphoma (ML) sets of large and small lymphocytes were compared separately.

Again, gray value sequences of seven measurements in the nuclear area were examined. For the set of small lymphocytes the following classification matrix resulted.

	LA	ML
LA	100%	
ML	12%	88%

with the average classification likelihood

	LA	ML
LR	9.66:1	1.60 × 10[illegible]:1

The set of large lymphocytes resulted the classification matrix

	LA	ML
LA	88%	12%
ML	32%	68%

with an average classification likelihood of

	LA	ML
LR	5.54:1	3.10 × 10[illegible]:1

It should be noted that large lymphocytes from the cases of benign lymphadenitis and malignant

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Carl Zeiss, Oberkochen.

† Original program version written by M. Ronald Hershel, M.Sc. Optical Sciences Center, University of Arizona, Tucson, Arizona, U.S.A.

‡ A density function is actually evaluated, however since the program utilizes ratio of probability densities, an interval for determining probability would divide out.

lymphoma resemble each other more than the corresponding small lymphocytes, and that some of the large lymphocytes in malignant lymphoma constitute signs of lymphoid tissue reaction without malignant transformation

DISCUSSION

The arrangement and distribution of chromatin in the nucleus, and or the uniformity vesicular or granular nature of cytoplasm give important diagnostic clues.

Multivariate analysis of gray values (MVA) described here depends largely on granularity in the scanned area. When evaluating the high average classification likelihoods found for the method introduced here one should consider that in both types of clinical material it would not be easy even for a trained human observer to make a cytodiagnostic decision on a cell-to-cell basis, with adequate reliability Furthermore all classification decisions were made on the basis of observing *a short scan line of 3 5 μ length through the center* of each cell only It is possible to read value sequences shorter than 7 values or longer The *restriction to seven values was chosen for reasons of computational efficiency*

The MVA program computes a number of other discriminators not utilized here, such as the variance of a value sequence, and the radius of gyration, which estimates the tendency of the absorbing material to be concentrated at the cell center or to be displaced towards the periphery of the nucleus After further field testing, the MVA program will be incorporated as a reducing subroutine in the TICAS master program.

It should be mentioned that the approach taken here is no longer computationally feasible when the dependency of a large number e.g., twenty gray values, are estimated. New methods capable of computing a likelihood for classification on the basis of large and even two *dimensionally extended* gray value dependence are more powerful for the detection of small differences, and are presently *undergoing testing*[4]

SUMMARY

A computer *program* is described which was designed to assess the multivariate distribution of gray values in cells of different types after measurement with a scanning microscope The program was applied to clinical material of hematopoetic origin and the results of the computerized image analyses of these cells are presented.

APPENDIX

The MVA program considers the multivariate distribution of consecutive gray values The underlying computations can readily be demonstrated using a model considering only two consecutive gray values, i.e., a bivariate distribution, and its covariance.

Let the first of the two considered gray values be denoted as X_1 and the value following it as X_2 *Let it furthermore* be assumed that both follow normal distributions, with means $\bar{X}_1$ and $\bar{X}_2$ and variances s_1^2 and s_2^2

If the value of X_2 is now completely independent of the value previously found for X_1 the bivariate distribution might look as shown in Figure 1 The elliptical figure indicates a contour of equal probability density

The ellipticity is determined by the respective variances s_1^2 and s_2^2 Since X_1 and X_2 are independent random variables the covariance is zero the two axes of the ellipse in Figure 1 are parallel to the X_1 and X_2 axes and the covariance matrix looks like this

$$\Sigma = \begin{bmatrix} s_1 & 0 \\ 0 & s_2 \end{bmatrix}$$

Should however the value for X_2 depend on the value of the preceding X_1 the bivariate distribution might look as shown in Figure 2. The covariance matrix in this case would look like this

$$\Sigma = \begin{bmatrix} s_1 & \mathrm{cov}_{1,2} \\ \mathrm{cov}_{2,1} & s_2 \end{bmatrix}$$

The covariance matrix is two-dimensional and has, therefore, two eigenvectors. They represent the directions of the axes of the ellipse in Figure 2

The covariance matrix is furthermore characterized by two "eigenvalues." These determine the ratio of one semi-axis to the other i e the ellipticity Both eigenvectors and eigenvalues can be computed from the covariance matrix by

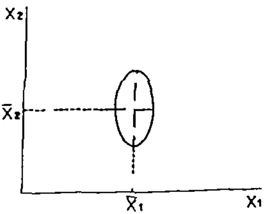

FIG 1 Bivariate distribution of two independent random variables Eigenvectors of the *covariance* matrix parallel the X_1 and X_2 axes.

standard methods I the case of bivariate distribution this requires only the solution of quadratic equation. For covariance matrices of higher dimensionality computer programs must be used, and are routinely available.

Let it now be assumed that the dependence of two consecutive gray values is different in cell images from cells of different type. The dependence of each cell type is described by its covariance matrix, and might be quite different as shown, for example, in Figure 3

Let now cell be observed with values X and X as indicated by the small triangle in Figure 3 and decision has to be made whether this point belongs to the bivariate distribution I rather than the bivariate distribution II It lies closer to the contour around distribution I but then, the probability density of I falls off much more rapidly than that of distribution II

It is not appropriate to estimate whether the point came from distribution I or II by computing the probabilities to have found value X as observed, and of X as observed from the marginal distributions for X_1 and X_2 since these are not independent, or stochastically separable

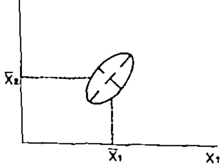

Fig 2 Bivariate distribution of two correlated random variables with positive covariance

Two possibilities now exist for arriving at solution. One may directly compute the probability density for I t the point, and that for population II and assign the unknown point to the set for which the probability density is higher Since the covariance of X and X has to be taken into account this requires an inversion of the covariance matrix, procedure which is not difficult for two dimensional example, but which is computationally expensive when a multivariate case is considered Most large computers have inversion routines available as system functions

The second possibility is to express the position of the point in terms of pair of new random variables which are independent. Such coordinate system are the eigenvectors of the covariance matrix

The new coordinates of the point are Z nd Z formed by linear combination of the original coordinates X and X (Fig 4)

$$Z = \quad X + \quad X$$
$$Z = b\,X + b_{2}X$$

Since Z and Z are independent random ariables, the probability density that the poi t is sample from population I can simply be computed as the product of probability densities to find the observed value of Z and to find the observed value of Z This probability density can then be compared to an analogously computed value for the new coordinate system based on the eigen vectors of set II

The transition to the new coordinate system is called linear transformation of the covariance matrix, and since it should result in setting all covariances to zero, it is lso called diagonalizing the covariance matrix

The eigenvalues represent the variances in the directions of the eigenvectors, or of the new inde

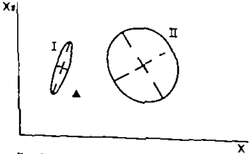

Fig 3 Contours of equal probability density for two bivariate distributions ith different covariance matrices

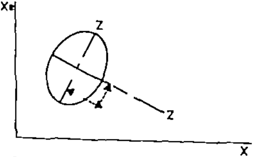

Fig 4 The coordinates of point may be expressed in terms of new random ariables Z_1 and Z_2 which are independent. Each represents linear transformation of the original correlated random variables X_1 and X

pendent random variables which were formed from linear combinations of the original gray values

The covariance matrices estimated from training sets for longer sequences of gray values, are represented by hyper-ellipsoids in a space of dimensionality as determined by the length of the considered sequence. Seven consecutive gray values represent a point in a seven dimensional space the covariance matrix for such a population has seven eigenvectors and eigenvalues.

The MVA program finds the eigenvectors and eigenvalues of these covariance matrices and directly computes a likelihood ratio that a cell belongs to a certain set, or another for which the covariance matrices are known. Cells are then assigned on the basis of maximum likelihood.

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Machine Characterization of Human Leukocytes by Acridine Orange Fluorescence

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Though the binding of the metachromatic fluorochrome acridine orange to nucleic acids has been extensively investigated and is well reviewed, treatment of its supravital uptake is less abundant. One study indicates that the metachromatic red fluorescence is localized by living cells in cytoplasmic particles also containing lysosomal enzyme (acid phosphatase) Green nuclear fluorescent staining is not held to be absolutely specific for DNA.

We here demonstrate that the metachromatic red fluorescence imparted supravitally to the granules of human leukocytes by acridine orange allows the leukocytes to be classified in three distinct groups. We also show that green fluorescent nuclear staining can be constant for cells of diverse function and nuclear morphology

In applying the techniques of microscope fluorimetry to acridine orange-stained live cells, we have been aware that the deeply saturated green and red fluorescences fade rapidly under excitation, that local variations in stain concentration cause variability in stain uptake that stain uptake may vary during the course of a series of measurements, and that vagaries of the exciting light source and photometer render accurate photometry all but impossible

Multichannel fluorometric analysis of single cells flowing in suspension[1] combined with argon-ion laser excitation at 488 millimicrons[2,4] provide the basis of an instrument[3] which avoids the above difficulties and allows populations of fluorescent-stained cells in suspension to be analyzed for per cell fluorescence at two wavelengths concurrently at rates up to 5,000 cells per second Cells are transported through a focused laser beam, and fluorescence within two wavelength ranges (green—5300 Å to 5800 Å, and red—6300 Å to 7000 Å) is simultaneously registered with two appropriately filtered photomultiplier tubes. The two pulses (approximately 10 μ seconds in duration) for each cell are amplified and determine the coordinates of a dot on a cathode ray tube The amplitude of cellular red fluorescence determines horizontal displacement, and green fluorescence determines vertical displacement. The cathode ray tube dots produced by many cells may be photographed to produce a scatter diagram as shown in Figure 1 In addition, the instrument incorporates the ability to generate an 80-step histogram of the distribution of dots along the horizontal axis. Such a histogram of the distribution of red-fluorescence pulse amplitudes is given in Figure 2.

The figures (1 and 2) are negative reproductions of photographs taken of the cathode ray tube when normal fresh human blood, diluted 1 part blood with 25 parts acridine orange solution (1 microgram of acridine orange per ml in 0 85% NaCl buffered with 005 M phosphates at pH 7 4) was run through the machine Three clusters of cathode ray tube dots are apparent (Fig 1)

Using a system of pulse amplitude discriminators and counters in the instrument, it has been possible to confirm by comparing machine counts with manual counts on a variety of normal blood samples that the cluster on the left comprises lymphocytes, and that on the right comprises granulocytes The middle cluster appears to be composed primarily of monocytes.

The microscopic appearance of live leukocytes stained with acridine dyes has been elsewhere described[5] and had suggested to us that mononuclear and granulocytic cells would be fluorometrically distinguishable by means of the red fluorescence of the granulocyte cytoplasmic contents and that leukocytes could be distinguished from erythrocytes by the green nuclear fluorescence However the emergence of the middle group was a surprise to us. At the concentration of acridine orange used here (10^{-6}) the fluorescence of red cells, platelets and reticulocytes is not detected. Sufficient staining for fluorescence microscopic examination is obtained at acridine orange concentrations of about 10^{-4} Lymphocytes show green nuclei and occasional fine points of cytoplasmic red. The polymorphous nuclei of granulocytes including eosinophils are green, and granulocyte cytoplasms are filled with red-fluorescing granules which, in the case of eosinophils, are coarser and may take a scarlet hue Some mononuclear cells have more red granules than do lymphocytes—we feel it is these cells which constitute the middle group

The mechanism by which the stain is taken up is not known to us. Mucopolysaccharides are

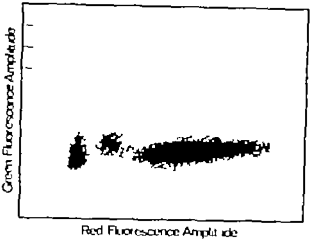

Fig. 1. Three clusters generated by acridine orange-stained human fresh blood. Each point corresponds to a single leukocyte. Green fluorescence amplitude is indicated on the vertical axis, and red fluorescence on the horizontal axis.

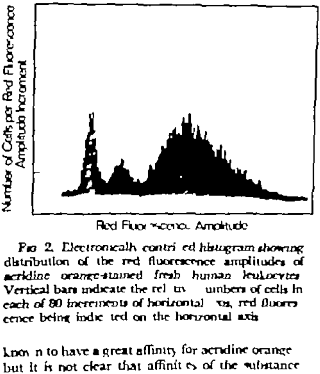

Fig. 2. Electronically controlled histogram showing distribution of the red fluorescence amplitudes of *acridine orange-stained fresh human leukocytes.* Vertical bars indicate the relative numbers of cells in *each of 80 increments of horizontal axis,* red fluorescence being indicated on the horizontal axis.

known to have a great affinity for acridine orange but it is not clear that affinities of the substance of the leukocyte granules are responsible for the observed high uptake of acridine orange and the attendent development of red metachromasia. Leukocytes fixed overnight in formalin, washed and stained in 10^{-4} acridine orange showed green nuclei but not the red cytoplasmic fluorescence. *Concentration of acridine* orange in the particles of cultured cells has been shown by Robbins and Marcus to be metabolism dependent. Since the granules may act as "sinks" for the dye it is not necessary to postulate an active process, such as pinocytosis, to explain the apparent high concentrations of dye attained *within the cell.* We know of no evidence that acridine orange exists in solution as particles capable of stimulating pinocytosis. Conceivably active deposition of the dye in the granules (even assuming no active cell uptake of dye) could constitute the basis of an aspect of the cleansing function of leukocytes. At any rate, it *appears that the differentiation according to red fluorescence shown in the figures reflects the functional differences between the leukocytes.* Studies elsewhere in progress, and soon to be reported seek to confirm the correspondence of these functional categories with the conventional morphologic categories, and to evaluate the clinical utility of this technique.[6]

We feel that the equivalence of the green fluorescence of *mononuclear and polymorphonuclear* cells is interesting in that it signifies a degree of *stoichiometry* between acridine orange green fluorescence and nuclear DNA-quantity and also indicates the relative absence of distributional errors. Other results in this laboratory indicate this technique may be useful for ploidy determination as well as for differential leukocyte counting.

SUMMARY

Three classes of leukocytes can be distinguished *in human whole blood samples supravitally stained* with acridine orange by a two-wavelength rapid fluorimeter.

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The Incidence of False Negative Cervical Cytology as Determined by Colposcopically Directed Biopsies

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Since the advent of the Papanicolaou smear for the purpose of cervical cancer screening there has been a continuing debate in the literature as to the actual efficiency of this method in detection of cervical neoplasia.[1,3-5,7,8] Estimates of the false negative rate of the Papanicolaou smear have ranged from 1.4[5] to 30 per cent.[1] Most of these estimates are based upon patient follow-up and the subsequent development of neoplasia in patients who were determined to be cytologically negative at the time of the initial screening. In order for the false negative rate of the cytology screening technique to be determined at the time of the initial screening it would be necessary for each cervix to be evaluated histologically at that time. However since random four-quadrant biopsies may miss a lesion already present on the cervix these biopsies must be colposcopically directed if meaningful data are to be obtained. Only two retrospective studies have compared cytology and colposcopy. False negative rates of 0.4 per cent[3] and 1.2 per cent[6] for carcinoma in situ were found. False negative rates for dysplasia were not determined. For this reason a prospective study comparing colposcopy and cytologic screening has been carried out in order to determine the incidence of cytologically negative cervical neoplasia.

Material and Methods

The patients reported in this study were clinic patients at Harbor General Hospital. In a prospective fashion each patient was cytologically and colposcopically* screened. The Papanicolaou smears were taken from the area of the squamocolumnar junction with a Milex® plastic spatula.† At the time of initial cytologic screening the colposcopic lesions were recorded. When the Papanicolaou smear had been reported as negative with no evidence of dysplastic or neoplastic cells present, the cervix was biopsied in the area demonstrated to be most abnormal by colposcopic visualization. A total of 100 patients were screened prospectively by this combination of cytology and colposcopy.

Results

By selection negative cytology was found in all patients (Table I). Colposcopically directed biopsies revealed three patients with mild dysplasia and one with mild to moderate dysplasia (*Figs. 1 and 2*). This indicates a cytologic false negative rate of 4 per cent for early squamous dysplasia. No instances of intraepithelial carcinoma were found.

Discussion

The incidence of cervical false negative cytologic evaluation has been a subject of controversy for some time. Most studies have been hampered by the need for continued patient follow-up after initial cytologic screening. In this study the colposcope has been used as an adjunct to the study of the incidence of cytologically negative cervical neoplasia. This instrument has the advantage of being able to localize areas which are abnormal and therefore which require cervical biopsy. None of the lesions which were biopsied in this series were visible gross and no other physical findings indicated the need for biopsy except for the abnormalities localized on colposcopic examination. From the biopsies of these 100 patients it is evident that the early dysplastic changes of the cervix are rarely missed by the technique utilized in our clinic for obtaining cervical smears. The use of a plastic

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The authors wish to express their gratitude to Mrs. Jimmye Mason for her diligence in the conduct of this study.

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* Colposcopes supplied by Leisegang Colposcope, Colposcope of America, Inc. P. O. Box 473, Kendall, Florida 33156.

† Milex Corporation, 5930 Northwest Highway Chicago, Illinois.

TABLE I

No. of times	Cytologic interpretation	Histologic interpretation
96	Negative	Negative for dysplasia or carcinoma
4	Negative	Squamous dysplasia mild (3) Squamous dysplasia, mild to moderate (1)

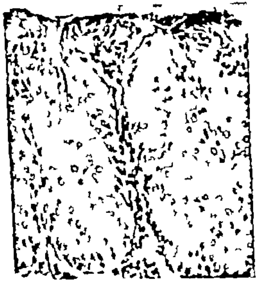

FIG 1 Cervical biopsy showing mild dysplasia (H & E, × 214)

spatula, which facilitates sampling of the squamocolumnar junction regardless of its exocervical or endocervical location, allows for abnormal cells to be exfoliated directly and placed upon the slide. Therefore, a more representative and consistent sample is obtained. By the utilization of this cytologic sampling technique in 100 patients no cervical lesion worse than mild to moderate dysplasia was found to be cytologically negative.

It may be concluded that when the cervical cytologic specimen is obtained in this fashion the incidence of false negative cytologic smears is very low.

Summary

A series of 100 patients with known normal cytologic smears but with abnormal colposcopic findings were biopsied in an attempt to determine the incidence of false negative cytologic evaluation. It was demonstrated that when the cervical scrape technique is used to obtain the cytologic specimen the incidence of cytologically negative cervical dysplasia was very low. A 4 per cent incidence of mild or mild to moderate dysplasia was found. There were no cases of intraepithelial carcinoma.

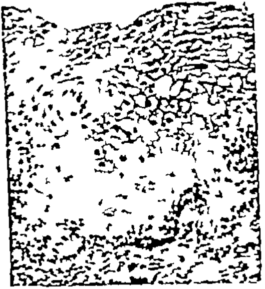

FIG 2 Cervical biopsy showing mild to moderate dysplasia (H & E, × 214)

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The Incidence of False Negative Cervical Cytology as Determined by Colposcopically Directed Biopsies

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Since the advent of the Papanicolaou smear for the purpose of cervical cancer screening there has been a continuing debate in the literature as to the actual efficiency of this method in detection of cervical neoplasia[1, 3, 4, 7, 8] Estimates of the false negative rate of the Papanicolaou smear have ranged from 1.4[3] to 30 per cent.[1] Most of these estimates are based upon patient follow-up and the subsequent development of neoplasia in patients who were determined to be cytologically negative at the time of the initial screening. In order for the false negative rate of the cytology screening technique to be determined at the time of the initial screening it would be necessary for each cervix to be evaluated histologically at that time. However since random four-quadrant biopsies may miss a lesion already present on the cervix these biopsies must be colposcopically directed if meaningful data are to be obtained. Only two retrospective studies have compared cytology and colposcopy. False negative rates of 0.4 per cent[2] and 1.2 per cent[6] for carcinoma in situ were found. False negative rates for dysplasia were not determined. For this reason a prospective study comparing colposcopy and cytologic screening has been carried out in order to determine the incidence of cytologically negative cervical neoplasia.

MATERIAL AND METHODS

The patients reported in this study were clinic patients at Harbor General Hospital. In a prospective fashion each patient was cytologically and colposcopically* screened. The Papanicolaou smears were taken from the area of the squamocolumnar junction with a Milex® plastic spatula.† At the time of initial cytologic screening the colposcopic lesions were recorded. When the Papanicolaou smear had been reported as negative with no evidence of dysplastic or neoplastic cells present, the cervix was biopsied in the area demonstrated to be most abnormal by colposcopic visualization. A total of 100 patients were screened prospectively by this combination of cytology and colposcopy.

RESULTS

By selection negative cytology was found in all patients (Table I). Colposcopically directed biopsies revealed three patients with mild dysplasia and one with mild to moderate dysplasia (*Figs. 1 and 2*). This indicates a cytologic false negative rate of 4 per cent for early squamous dysplasia. No instances of intraepithelial carcinoma were found.

DISCUSSION

The incidence of cervical false negative cytologic evaluation has been a subject of controversy for some time. Most studies have been hampered by the need for continued patient follow-up after initial cytologic screening. In this study the colposcope has been used as an adjunct to the study of the incidence of cytologically negative cervical neoplasia. This instrument has the advantage of being able to localize areas which are abnormal and therefore which require cervical biopsy. None of the lesions which were biopsied in this series were visible grossly and no other physical findings indicated the need for biopsy except for the abnormalities visualized on colposcopic examination. From the biopsies of these 100 patients it is evident that the early dysplastic changes of the cervix are rarely missed by the technique utilized in our clinic for obtaining cervical smears. The use of a plastic

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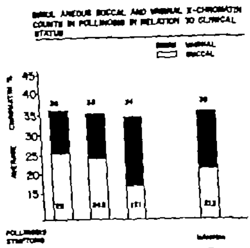

FIG. 1 The X-chromatin frequency decreases in buccal but not in vaginal cells during active pollinosis.

per cent with mild symptoms but with more severe symptoms the buccal count dropped to 17.1 per cent, a difference significant beyond the .01 level of confidence. Buccal counts taken at the time of an infection appeared to be somewhat higher than counts taken when hay fever was moderate but less than counts in patients with mild hay fever or no symptoms.

Counts from patients who were sampled two or more times were examined to see whether counts shifted from one time to the next in any consistent way. Sets of such successive counts were obtained in 53 instances from 32 hyposensitized atopic patients. Buccal and vaginal counts varied together 19 times, remained unchanged four times and moved in opposite directions in 12 instances. Changes in X-chromatin counts in the oral and vaginal cavities therefore appear to be unrelated to one another.

Successive changes in sets of counts were examined in patients with active ragweed hay fever symptoms. Buccal counts dropped in six of seven instances when symptoms increased in severity. Vaginal counts showed no trend. In nine cases with decreasing symptoms, buccal counts rose in six and fell in three; vaginal counts rose in five and dropped in four instances. Eight sets of paired buccal and vaginal counts taken during an upper respiratory or systemic infection were compared with counts taken in the same patient days to weeks earlier or later. In six of the eight instances the buccal count rose at the time of initial infection and five of the eight vaginal counts also rose.

DISCUSSION

The X-chromatin, also known as the Barr body after its discoverer, is a single intranuclear DNA particle believed to be one of the two female sex chromosomes. In squamous epithelial cells it is located near the inner surface of the nuclear membrane at the disc edge. Lowered counts would be produced if this particle shifted from its characteristic site in the nucleus or if it unfolded and thus became invisible as it participates in dynamic changes in the nucleus. Another possibility is that antigen-antibody complexes alter the staining property of DNA material. We have excluded the possibility that lowered X-chromatin counts are due to pre-mitotic changes, because these superficial cells from a stratified squamous epithelium are terminal and not destined to reproduce themselves.

Vaginal and buccal X-chromatin counts both vary from time to time in the same individual. Buccal variations can be correlated with hay fever symptoms as shown in this and in a previous study in which an asymptomatic group served as controls. Thus it appears that active allergic symptoms are accompanied by a cytologic effect which penetrates to the interior of the nucleus. Altered physiology of DNA material is probable since the position or physical state of the X-chromatin is altered. From this work it is impossible to state whether a cytoplasmic change such as breakdown in lysosomes has instigated the nuclear change or whether the nuclear change represents a primary target in the atopic reaction.

The fact that vaginal and buccal counts in the same individual do not always vary in the same direction suggests that the changes were not precipitated by systemic factors such as hormone changes or a shift in acid-base balance. It appears that the factor producing decrease in buccal X-chromatin counts in allergic rhinitis is relatively localized.

Our finding that the buccal counts are lower than vaginal counts is in agreement with Moore who took issue with Sohval[6] on this point. This discrepancy may however only stem from clinical factors operative at the time the specimens were collected. Work reported in another communication[4] has demonstrated that local effects of several irritants introduced into the buccal cavity alters X-chromatin counts within two minutes. Lower buccal counts may well result from the introduction of such substances as air, smoke, liquids and solids into the buccal cavity while the vagina is relatively free of such influences. The buccal mucosa must with great frequency contend with wide variations in pH, osmolarity, temperature, chemical composition and friction. This suggests that the X-chromatin negative cells either survive longer than the X-chromatin positive cells in the face of irritation or that X-chromatin positive cells adapt to irritation by becoming X-chromatin negative.

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Comparison of X-Chromatin Frequency in Buccal and Vaginal Cells from Atopic Women

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Buccal X-chromatin counts have been found to drop in ragweed sensitive women during ragweed season when symptoms were active.[2] The intensity of rhinitis was found to correlate inversely with X-chromatin frequency in buccal cells. Hyposensitized women who were free of symptoms through a pollen season failed to show depression of buccal X-chromatin counts at the height of ragweed season. If pollinosis affects buccal mucosa through a systemic mechanism we would expect that vaginal X-chromatin counts would parallel those of the buccal cells. This study of simultaneously collected vaginal and buccal cells reveals that buccal X-chromatin counts may vary independently of vaginal counts.

METHOD

Buccal and vaginal specimens from atopic women were collected on the same day. Most of these specimens were collected on hyposensitized ragweed sensitive women during and after the 1963 ragweed pollen season. The buccal specimen was obtained by scraping the inner cheek with the smooth rolled edge of a plastic spoon handle. This was immediately placed in a bottle of 50% ethyl alcohol, from which cells were recovered later by centrifugation. Smear preparation, Papanicolaou stain, and reading methods are described elsewhere.[4] X-chromatin counts represent the number of cells containing X-chromatin per 100 countable cells. The countable cell is an intact cell which has a vesicular nucleus. Variation in duplicate readings of slides by the two thoroughly experienced technician-observers who performed this study averaged 2.66 per cent for 50 vaginal slides and 2.71 per cent for 200 buccal slides. Vaginal cells were collected by placing a small cotton tampon ("Fibs" by Kotex) in the vagina for 2 to 4 hours prior to taking the buccal specimen. The tampon was placed in a bottle of 50% ethyl alcohol from which the cells were recovered and processed in a manner identical to those from the buccal mucosa. Since no significant difference was found in counts taken on vaginal scrapings compared with parallel tampon collection specimens, the latter technic was chosen for its convenience.

One hundred eight pairs of smears which were technically satisfactory for evaluation were obtained on 52 atopic women from a single private allergy practice. Symptoms were noted on the patient's chart at the time each specimen was taken. Both buccal and vaginal specimens were counted by the same technician. The technician was thoroughly experienced in this technique and had no knowledge of the patient's symptoms. The intensity of pollinosis symptoms was classified by the clinician without reference to X-chromatin counts.

Mild hay fever, rated one plus, was intermittent and readily controlled by an occasional antihistamine. Moderate to severe hay fever, rated two plus, required frequent use of antihistamines or topical corticosteroids with good to poor relief. Infections were diagnosed on the basis of epidemiology, fever, purulent nasal discharge or sputum and malaise. Sometimes muscle and joint aching or gastro-intestinal disturbances accompanied these events.

RESULTS

Vaginal counts are usually higher than buccal counts. Of 108 pairs of counts only 9 buccal counts exceeded the vaginal count and in another 6 instances counts were equal. Thus vaginal counts were higher than buccal counts in 93 instances. The average X-chromatin count for buccal cells was 23.1 per cent and for the almost simultaneously collected vaginal cells was 34.4 per cent. The range for the buccal counts was 9 to 58 per cent and for vaginal counts was 9 to 60 per cent.

Figure 1 shows average buccal and vaginal counts in pollinosis patients grouped by symptom intensity. It may be readily seen that there is virtually no fluctuation in average vaginal counts related to intensity of symptoms. Buccal counts show an insignificant decrease from 25.5 to 24.2

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In Vitro Observations of Sex Chromatin in Cells from the Human Uterine Cervix

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A wealth of clinical data concerning the sex chromatin body has been amassed since first described by Barr and Bertram. A few of the basic questions have been answered, but much pertinent information is still speculative. The sex chromatin mass consists of single heterochromatic X-chromosome. This heterochromatic chromosome replicates late in the S-phase. It is not present in early embryonic tissues[4, 14] but appears later as one X-chromosome becomes heterochromatic in seemingly random manner.[8, 10] Originally this heteropyknosis was thought to indicate state of genetic inactivation, but this simple explanation does not appear to be the entire answer.[10, 15] In order to elucidate the question of the activity and function of the heterochromatic X-chromosome, it seemed important to determine whether this body was present throughout the entire interphase.[13, 14] This study was designed to observe the sex chromatin *in vitro* throughout the whole mitotic cycle in human cells.

Materials and Methods

All studies were performed on cells obtained from the adult human uterine cervix. Biopsies were taken of clinically normal cervices of patients in the out-patient clinic or operating rooms. The tissue was rinsed in several changes of balanced salt solution to remove as much mucus and blood as possible. The epithelium was separated from the stroma under dissecting microscope, cut to 1 mm fragments, and cultured on # 1 thickness coverslips. The stroma was placed in separate culture containers. Half of the biopsy material was fixed in 10 % formalin for histologic preparation. The fluid medium consisted of Eagle's MEM supplemented with 15 % fetal calf serum, 100 units/ml penicillin and 100 mcg streptomycin, the gas phase was 5 % CO_2 in air.[10, 11] Cultures were maintained at 37° C. Only primary explants of the epithelium were used, but fibroblast cultures up to 4 passages were utilized. Time lapse photographs were taken at various intervals from 8–60 seconds with phase optics at × 1000. Single cells or small groups of cells were followed up to 72 hours. After completion of the cinematography the cultures were fixed and stained with the Feulgen technique.

In this study the sex chromatin was defined as mass of chromatin material measuring 1 to 2 μ in diameter adjacent to the inner surface of the nuclear membrane. It was usually round or planoconvex. It appeared solid or as an inverted "u" shaped body with the open part against the nuclear membrane.[12]

Results

Initially cell undergoing mitosis was selected to be followed to the next mitosis. It was planned to observe the appearance and activity of the Barr body throughout interphase. Sex chromatin was found in approximately 25 per cent of these cells by phase contrast observations in living cultures and 1 35 per cent of the cells in fixed, stained cultures.

Two epithelial cells and 3 fibroblasts were identified mitosis (usually late prometaphase or metaphase). One or both daughter cells were followed from 4 to 48 hours. Two fibroblasts were followed through a second mitosis 28 hours later. No sex chromatin was observed at any time in these actively dividing cells (Tables I and II, and Fig. 1).

One epithelial cell that did not contain sex chromatin mass was observed to enter mitosis after 26 hours. One daughter cell was followed for 2 hours, and no sex chromatin was observed to form (Table II).

When no sex chromatin formed in cells observed from mitosis, cell which contained sex chromatin mass was selected for continued observation.

Sex chromatin was identified in 3 epithelial cells and 6 fibroblasts. The epithelial cells were followed for 7, 21 and 50 hours, and the fibroblasts from 15 to 59 hours (Tables I and II and Fig. 1). None of the cells containing sex chromatin

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Supported by Grant # DRG 868 from the Damon Runyon Memorial Fund for Cancer Research.

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SUMMARY

Vaginal and buccal X-chromatin counts could vary simultaneously in the same or in opposite directions on successive paired counts from these two areas. During the course of active hay fever buccal counts were depressed but not vaginal counts. The mechanism by which ragweed pollen depresses buccal X-chromatin counts in pollinosis appears to be primarily local rather than systemic. The vaginal X-chromatin count was higher than the buccal count in 86 per cent of 108 simultaneously gathered pairs of specimens. It is suggested that an increased local surface irritation in the mouth accounts for this difference

ACKNOWLEDGMENTS

This work was supported by U S P H S Grant #GM 11886 to Howard University We thank Mrs. Lorraine Miner and Miss Beatrice Vargas for technical assistance

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In Vitro Observations of Sex Chromatin in Cells from the Human Uterine Cervix

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A wealth of clinical data concerning the sex chromatin body has been amassed since first described by Barr and Bertram A few of the basic questions have been answered, but much pertinent information is still speculative. The sex chromatin mass consists of a single heterochromatic X-chromosome This heterochromatic chromosome replicates late in the S-phase It is not present in early embryonic tissues[1, 14] but appears later as one X-chromosome becomes heterochromatic in seemingly random manner [26] Originally this heteropyknosis was thought to indicate state of genetic inactivation, but this simple explanation does not appear to be the entire answer [16, 19] In order to elucidate the question of the activity and function of the heterochromatic X-chromosome, it seemed important to determine whether this body was present throughout the entire interphase [6, 11]. This study was designed to observe the sex chromatin in vivo throughout the whole mitotic cycle in human cells

MATERIALS AND METHODS

All studies were performed on cells obtained from the adult human uterine cervix Biopsies were taken of clinically normal cervices of patients in the out-patient clinic or operating rooms. The tissue was rinsed in several changes of balanced salt solution to remove as much mucus and blood as possible The epithelium was separated from the stroma under dissecting microscope cut to 1 mm fragments, and cultured on # 1 thickness coverslips The stroma was placed in separate culture containers Half of the biopsy material was fixed in 10 % formalin for histologic preparation The fluid medium consisted of Eagle MEM supplemented with 15 fetal calf serum, 100 units/ml penicillin and 100 mcg streptomycin the gas phase was 5% CO_2 in air [22, 24] Cultures were maintained at 37° C Only primary explants of the epithelium were used, but fibroblast cultures up to 4 passages were utilized. Time lapse photographs were taken at various intervals from 8–60 seconds with phase optics at × 1000. Single cells or small groups of cells were followed up to 72 hours After completion of the cinematography the cultures were fixed and stained with the Feulgen technique

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RESULTS

Initially cell undergoing mitosis was selected to be followed to the next mitosis It was planned to observe the appearance and activity of the Barr body throughout interphase Sex chromatin was found in approximately 25 per cent of these cells by phase contrast observations in living cultures and in 35 per cent of the cells in fixed, stained cultures

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Fig. 1 Two sequences of photographs prepared from cinematography films of human cervical fibroblasts, phase contrast, × 650 a, b, Photographs of cell observed to contain Barr body t all times during 30 hours of observation. N mitosis occurred. Photographs 0 hours ( ) and 50 hours (b) c, d, Photographs of cell observed during the entire period from one mitosis to the next (28 hours metaphase to metaphase) No Barr body was observed. Photographs at 4 hours (c) and 21 hours (d)

TABLE I

Schematic Representation of Length of Time Cells in Cervical Fibroblast Culture Observed

(M = mitosis)

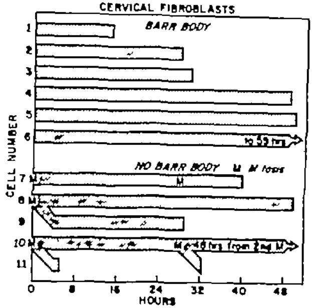
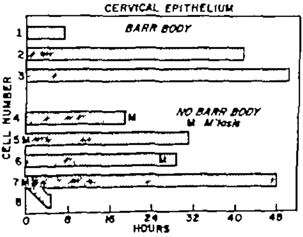


TABLE II

Schematic Representation of Length of Time Cells in Cervical Epithelial Cell Cultures Observed

(M = mitosis)



was observed to enter mitosis although the usual generation time of these cells in our system is 24 to 30 hours. The sex chromatin remained stable throughout the period of observation.

Discussion

The sex chromatin mass was first described in unstained exfoliated cells by Silva Inaunza[17] and in tissue culture by Miles.[15] These were static observations.

James[13] was one of the first to relate the presence of sex chromatin to the mitotic cycle when he reported the corpuscle (Barr body) remains visible up to late prophase in the mitotic cycle without showing any essential changes in size and shape" in cultures of rabbit cells. He also commented that sex chromatin becomes distinguishable in the daughter cells early in telophase as the nuclear membranes become definite. He does not give precise details as to the number of such observations or how these were made. In a later study[14] utilizing a short exposure of tritiated thymidine, he found no radioactivity over the sex chromatin and by Feulgen cytophotometry he noted that there were a few cells in mid to late S-phase that contained sex chromatin. From these observations he concluded that "the heteropyknotic X-chromosome decondenses somewhere in the middle of the S-phase undergoes replication and subsequently recondenses " He described a small sex chromatin mass before replication and a larger one afterward.

DeMars[7] definitely correlated the sex chromatin seen in living cells to that seen in stained cells. He identified a cell with sex chromatin under phase contrast the culture was then fixed stained, and the same cell again observed Twenty-seven such observations from cultures of human skin fibroblasts confirmed that the stained and living sex chromatin was identical. In "several hundred observations" he noted that cells in early interphase lacked a clearly defined sex chromatin mass, but noted it to be present in the "last few hours" prior to the next division. He apparently did not use cinematography in these observations. In a later report he comments that the sex chromatin in human fibroblasts was found in daughter cells only 16 or more hours after mitosis (in a 20–24 hr total generation time) He hypothesized that the typical sex chromatin is a post-replication form of the X-chromosome

Hosli[12] presented an interesting hypothesis on the shape and position of the sex chromatin of cultured human embryo cells in a preliminary report. He differed with DeMars in that he observed a form of the sex chromatin body in early interphase and a more dense type in late interphase The sex chromatin was followed into mitosis but was not observed continuously in early *interphase He comments that more* definitive studies are in progress

Schwarzacher[16] described cinematographic recordings of the sex chromatin Using cultures of human skin fibroblasts he observed eleven cells from 7 to 52 hours Nine cells contained sex chromatin bodies. None of these was observed in mitosis The sex chromatin *remained constant* during the period of observation One additional cell was observed to *undergo mitosis in which a* large questionable sex chromatin mass appeared in prophase and reappeared in only one of the daughter cells.

Mittwoch show photomicrographs of the remnants of sex chromatin mass in a fibroblast in prophase and possible reformation in telophase in one daughter cell.

In this study the sex chromosome remained present during the entire period of observation in all cells in which it was identified. None of these cells were seen to enter mitosis although the cells were followed twice the usual generation time. Sex chromatin was not found in the cells which were actively engaged in mitosis at any time during interphase between two observed mitoses or up to double the usual generation time prior to or following mitosis. The cell density was similar in all cultures.

Our data, though preliminary in numbers of observations, seems to agree with the cinematographic observations on human fibroblasts by Schwarzacher. These differ from the observations of James on rabbit and human cells, of DeMars on human adult fibroblasts, and of Holt[12] on human embryo fibroblasts as all observed the sex chromatin to be present in portions of interphase, but to be present also in cells entering active mitosis. Klinger has recently reported sex chromatin variation in vitro due to cell densities. Further observations on similar cells may resolve these differences.

A possible hypothesis for our observations may be that the sex chromatin is related to the mitotic activity or capabilities of the cell, it is only present in resting (perhaps differentiated) cells, not in reproducing cells. The percentage of cells containing sex chromatin is low in actively growing cultures during the logarithmic phase of growth. The sex chromatin is not present in early embryonic tissue or in more undifferentiated blood cells. The sex chromatin is also absent in most malignant cells and may be lost from tissue culture cells after several transplants. We have not identified sex chromatin mass in malignant cell lines isolated in our laboratory from an adenocarcinoma of the cervix, adenocarcinoma of the breast and pelvic hemangiopericytoma, but the mass is present in cells from carcinoma in situ of the cervix. Cyclic variation of the Barr body during menstrual cycles is still open to question. However it would seem that some functional activity is present the heterochromatic X-chromosome as indicated by glucose-6-phosphate studies in the mule. Replication may also occur while this X-chromosome is heterochromatic as indicated by tritiated thymidine incorporation.

Although far from the final answer regarding the activity and function of the Barr body these observations suggest possible correlation between the presence of the heterochromatic X chromosome and cellular differentiation.

SUMMARY

Time lapse cinematographic observations of epithelial cells and fibroblasts from the adult human uterine cervix showed the heterochromatic X-chromosome to be absent throughout interphase in cells actively dividing, but to be present continuously in cells that were not observed to divide. This suggests possible correlation between the presence of the heterochromatic X-chromosome and cellular differentiation. These findings are discussed light of other *in vitro* studies.

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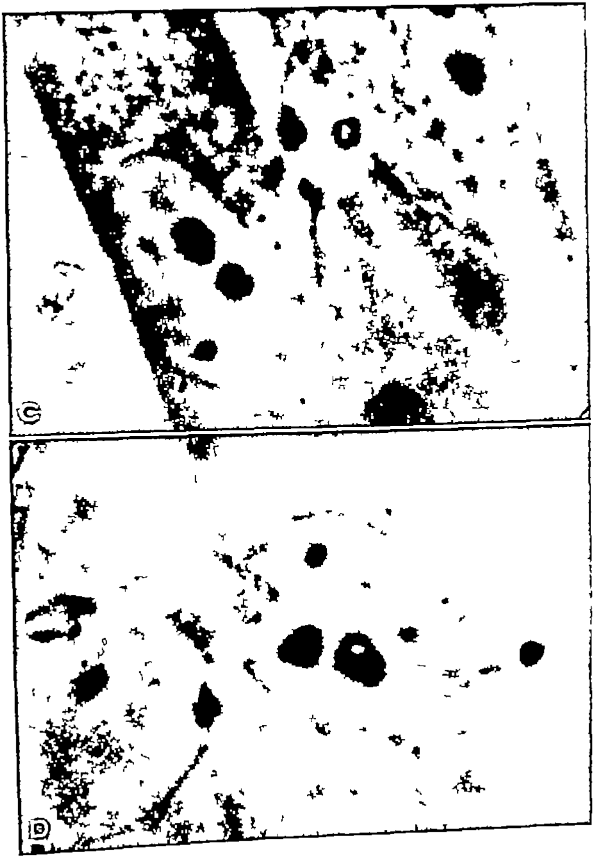

FIG. 1 C D

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Miltwoch[illegible] shows photomicrographs of the remnants of a sex chromatin mass i a fibroblast in prophase and possible reformation in telophase in one daughter cell.

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Our data, though preliminary in numbers of observations, seems to agree with the cinematographic observations on human fibroblasts by Schwarzacher[illegible] These differ from the observations of James[10, 11] on rabbit and human cells, of DeMars on human adult fibroblasts, and of Holz[12] on human embryo fibroblasts as all observed the sex chromatin to be present in portions of interphase, but to be present also in cells entering active mitosis Klinger[illegible] has recently reported sex chromatin variation *in vitro* due to cell densities Further observations on similar cells may resolve these differences

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Although far from the final answer regarding the activity and function of the Barr body these observations suggest possible correlation between the presence of the heterochromic X chromosome and cellular differentiation

SUMMARY

Time-lapse cinematographic observations of epithelial cells and fibroblasts from the adult human uterine cervix showed the heterochromic X-chromosome to be absent throughout interphase in cells actively dividing, but to be present continuously in cells that were not observed to divide This suggests a possible correlation between the presence of the heterochromic X-chromosome and cellular differentiation These findings are discussed in light of other *in vitro* studies

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Comet’ or Decoy Cells Found in Urinary Sediment Smears

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The results of the first systematic examination of urine sediment smears for the detection of tumors of the bladder were presented by Papanicolaou and Marshall in 1945[4] and Schmidlapp and Marshall[7] published further results in 1948 They concluded that the technique was successful for finding malignant bladder tumors but that it was of little value for indicating the presence of benign tumors, the so-called benign papillomata. Since that time the method has been utilized successfully on much larger scale for the detection of bladder tumors of occupational origin.

Papanicolaou had noted the presence of certain atypical cells in some smears to which he gave the name comet" cells, bec use of their shape and illustrated examples of them in his *Atlas of Exfoliative Cytology* Koss[3] also comments on these cells pointing out that M Andrew Ricci, the chief cytotechnologist of Memorial Hospital, New York, more appropriately named them decoy' cells

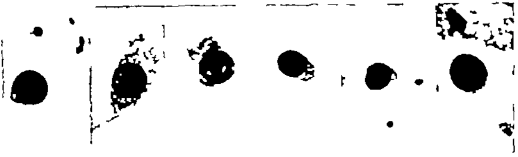

Fig 1 Six typical decoy cells, all exhibiting hyperchromatic nuclei, coarse chromatin pattern, and ery little cytoplasm Papanicolaou stain X 342.

These cells are very commonly found in voided urine smears and we have found them on innumerable occasions when carrying out mass screening of urine of workers in the dyestuffs and rubber industries where men have been exposed t bladder carcinogens in the past

The cells have many characteristics of malignancy and it is important that they should be recognized for what they are. Indeed, many experienced cytologists who may be unfamiliar with urinary tract cytology can mistake them for malignant cells and will report them as such Urine smears containing these cells have often been referred to me because no tumor had been found on cystoscopy

They are found in both male and female urine from healthy subjects as well as in urine containing malignant cells The cells themselves are ery characteristic. Figure 1 show six typical decoy cells They consist, for the most part, of round or oval nuclei with a coarse chromatin network or they may be completely hyperchromatic There may be a slight tag of cytoplasm attached to the nucleus or this may be completely absent as seen in Figure 2 Occasionally they may be binucleat (Fig 3) and they tend t vary in size somewhat Sometimes there may be only few such cells in whole smear or they may be fairly numerous, 30 to 40 per smear

It is not known from what tissue they originate As first it was suggested that they might be spermatogonia or arise from other prostatic or testicular tissue, but as they are also found in urine from the female this cannot be so I am of the opinion that they are transitional epithelial cells in the process of degeneration Due to some as yet unknow factor in the urine such as pH or osmotic pressure, the transitional cells start to shrink, the nuclei become pyknotic and the cytoplasm begins to disintegrate and finally disappears The nucleus

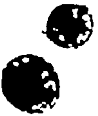

FIG. 2 Decoy cells without cytoplasm. Papanicolaou stain. × 640.

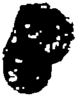

FIG. 3 Binucleate decoy cell Papanicolaou stain × 640

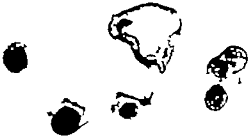

FIG. 4 Transitional epithelial cells beginning to degenerate and to form decoy cells. Papanicolaou stain. × 461

initially swells and finally disintegrates. Figure 4 shows transitional epithelial cells beginning to degenerate and forming decoy cells. Figure 5 shows a binucleate decoy cell with one of the nuclei beginning to disintegrate. Figures 6 and 7 show decoy cell nuclei shrinking and disintegrating.

Papanicolaou, Koss and ourselves have used the Papanicolaou method of preparation and staining, but these cells have also been noted in other methods of preparation and are particularly numerous in those made by the cell concentration

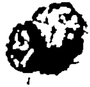

FIG. 5. Binucleate decoy cell with one of the nuclei undergoing degeneration Papanicolaou stain × 640.

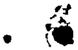

FIG. 6. Decoy cell with shrinking nucleus Papanicolaou stain. × 640.

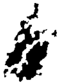

FIG. 7 Decoy cell with nucleus undergoing karyorrhexis. Papanicolaou stain. × 640.

methods such as that of Rofe[6] This may be due to excessive centrifugation which probably exacerbates the disintegration of the transitional cells

At best, urinary tract cytology is very difficult to interpret and the presence of comet or decoy cells can prove to be major pitfall. The purpose of this paper is to warn persons who are unfamiliar with this type of cytology or who are doing it for the first time to beware of these cells

Summary

Comet or decoy cells often found in urine smears, look rather like malignant cells and can mislead pathologists and cytotechnicians into giving positive report when the cystoscopy proves to be negative. They are only atypical transitional cells Their characteristics are illustrated and suggestion as to how they originate is offered.

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Peritoneal Fluid Cytology Associated with a Solid Ovarian Teratoma in a Mouse

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The treatment of ovarian cancer continues to be associated with poor patient survival due mainly to our inability to detect the disease in its incipient stages. Malignant solid teratomas are one type of ovarian cancer in which the prognosis is universally unfavorable.[6]

Spontaneous malignant ovarian teratomas in a mouse are histologically like the teratomas observed in women.[1, 7, 22] In normal women and in female mice, peritoneal fluid is in a state of cellular equilibrium. We have demonstrated that the distribution of peritoneal fluid cells in women and female mice is similarly affected by endocrine alterations, radiation and inflammation, as well as various physiologic states.[3-6, 9-11, 19] We believe that peritoneal fluid lends itself to cytodiagnosis. *Our previous studies indicated that the female* mouse is the laboratory model most comparable to the women, from the standpoint of peritoneal fluid cellular composition, and consequently, as it is difficult to examine closely a large group of women over an extended period of time for ovarian tumor formation, we have used the mouse.

We have found that primary ovarian neoplasms of coelomic (germinal) epithelium in mice were related with a notable change in the cytodifferential count of peritoneal fluid months before mor*phologic cellular variations were seen.*[7] *In another* study, we observed that ovarian neoplasms of specialized gonadal stroma induced by irradiation were usually associated with morphologic cellular changes in the peritoneal fluid months before shifting of the cytodifferential count of the peritoneal fluid.[18]

Primary solid malignant teratomas of the ovary probably arise from germ cells but how the germ cell accomplishes the teratoma formation remains unknown.[6] Histologically these neoplasms contain elements from the three germ layers and may be composed entirely of mature (adult) tissue or show a variable mixture of immature embryonic and mature tissues.

In the present investigation, we attempted to detect any changes in cellular morphology and distribution in peritoneal fluid associated with the *development of a solid ovarian teratoma in a* mouse.

MATERIALS AND METHODS

We divided 72 adult litter mate female mice of the C3B6F1 hybrids (18–20 gr) into groups according to genotypes W/w, W/w and w/w. All mice were individualized by ear identification. We *aspirated serous fluid from the abdominal cavity* of the mice each month for one year by using a No. 27 gauge needle attached to a 1 ml tuberculin syringe, introduced into the abdominal cavity with the animal in the supine position. Holding the needle firmly in place, we rotated the mouse back to its normal position so that fluid would drain toward the needle. All animals were held manually without squeezing and without using anesthesia or artificial restraining devices.[13] After placing the specimen on an albumin-coated slide, we fixed and *stained it according to the Papanicolaou method.*[17]

We studied each cytologic specimen for morphologic cellular changes as well as randomly counting 200 cells under microscopic oil immersion and grouped them as mesothelial cells, lymphocytes, polymorphonuclear leukocytes, histiocytes, mast cells and bare nuclei. We defined the cytodifferential count as the relative proportion or percentage of each cell present in peritoneal fluid.[1] When cells did not have cytoplasm, we termed them bare nuclei.

RESULTS

Of the 72 animals studied for one year, only one developed an ovarian neoplasm and that was a solid teratoma at 39 weeks of age. The ovarian teratoma occurred in an animal of the genotype w/w and was *0.4 x 0.5 cm in diameter. There was* no gross evidence of metastatic spread of the disease at the time of autopsy. Histologically, cari

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Reprint Requests: Department of Obstetrics and Gynecology, The George Washington University Medical Center, 2150 Pennsylvania Avenue, N.W., Washington, D.C. 20037 lage, keratin, skin, intestinal loops with goblet cells, alveoli with cuboidal epithelium, melanin, and brain tissue were found. Mitotic figures were numerous and malignant changes were observed in several areas of the ovary but no break in the outer lining of the ovary by tumor was seen.

Morphologic cellular changes in the peritoneal fluid appeared when the animal was 199 days old or 74 days before autopsy (Fig 1) Some of the cells, which called mesothelial cells, were larger than normal and their oval nucleus contained homogeneous nucleoplasm with minimal chromatin clumping and distinct nuclear membrane Other cells were interpreted as mesothelial cells, and had lobulated and often hyperchromatic nucleus and some chromatin clumping The cytoplasm may be vacuolated. At 229 days of age (44 days before autopsy) further progressive morphologic cellular changes in the peritoneal fluid of the mouse were found in the large cells with abundant granular and finely vacuolated cytoplasm w called mesothelial cells The nuclei contained minimal chromatin clumping with distinct nuclear borders Two weeks before autopsy cells with vacuolated cytoplasm, hyperchromatic and peripherally located nuclei as well as multiple bizarre-appearing cells were present in the peritoneal fluid (Fig 2) These latter cells in the peritoneal fluid of the mouse with the ovarian teratoma appeared to originate from the ovary (Fig 3) We did not see abnormal cellular alterations in the peritoneal fluid of the remaining tumor-free animals

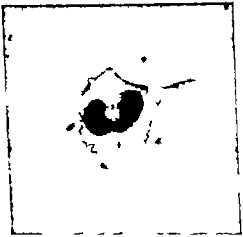

FIG 1 An enlarged mesothelial cell with bizarre bean shaped nucleus seen in the peritoneal fluid 74 days before autopsy The cytoplasm is granular and ery finely acuolated The cell border is not distinct, but retains somewhat round shape (×3000)

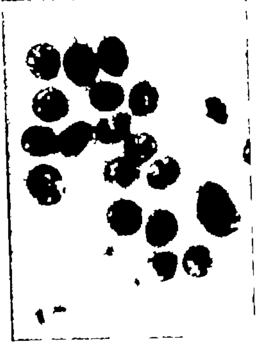

FIG. 2 Peritoneal fluid specimen obtained 2 weeks before autopsy contained cells with abundan cytoplasm and big nuclei uniform in size and evenly distributed chromatin clumping (×3000)

Of total of eight monthly serial cytodifferential counts of peritoneal fluid in the control group of mice and in the mouse with an ovarian teratoma, only when the animals were 37 weeks of age did the values of the teratoma mouse not fall within the range recorded for the controls in regard to mesothelial cells, lymphocytes and bare nuclei cells (Table I)

DISCUSSION

Spontaneous malignant solid teratomas of the ovary in mouse are extremely uncommon [illegible] and the study of peritoneal fluid cellular patterns during their neoplastic developement has not, to our knowledge, been reported Furth and Boon said there existed great similarity between ovarian neoplasms of women and mice.[4]

Benign morphologic cellular changes were first observed in the peritoneal fluid of the mouse with an ovarian teratoma slightly more than 10 weeks before autopsy Mesothelial cells exhibited the first morphologic variation, being 4 to 5 times larger than normal mesothelial cells We believe these large cells originated and were exfoliated from the surface epithelium of the ovary and represented an early response to neoplastic growth of the solid teratoma type within the ovary

Continued development of the ovarian teratoma was associated with more bizarre mesothelial cell changes in the peritoneal fluid. Cytologic specimens of the peritoneal fluid a few weeks before autopsy contained many different cells which were very similar to those observed in the teratoma. These cells were found in spite of histologic evidence of an intact serosa of the ovary. The exact mechanism by which these cells in the ovarian stroma escape to the peritoneal fluid is not known. The surface epithelium of the ovary appears to be permeable to either benign or malignant cells in the ovarian stroma. The significance of these cells in peritoneal fluid to aid in the earlier diagnosis of ovarian tumors or as a guide to therapy awaits further study.

Fig. 3. Tissue examination of the ovary following autopsy revealed cellular cells as seen in Fig. 2 (×1200).

Differences in the cytodifferential count of peritoneal fluid in the ovarian teratoma mouse as compared to control mice occurred only two weeks *before* autopsy of the tumor bearing mouse. The decrease in the relative proportion of mesothelial cells may be *accounted for by an increase in bare* nuclei. A severe alteration of the exfoliative process of the mesothelium secondary to the ovarian teratoma may have produced this observation. The decrease in lymphocytes may have reflected a lowered immunologic response of the mouse due to the neoplastic tumor.

Any method used for the early detection of primary ovarian neoplasms must take into consideration the histogenic classification of ovarian neoplasms. We have previously observed that ovarian neoplasms of specialized gonadal stroma (sex cords and mesenchyma) induced by radiation and resulting in prompt and well-developed primary granulosa cell tumors and luteomas are associated with morphologic cellular changes in the peritoneal fluid.[3] We have also demonstrated that primary ovarian neoplasms of coelomic (germinal) epithelium in mice were related to a notable change in the cytodifferential count of peritoneal fluid months before morphologic cellular variations were seen.[1] In this study an ovarian neoplasm of germ cell origin was associated with early and progressive morphologic changes of the mesothelial cells in the peritoneal fluid plus the appearance of cells which originated from the solid teratoma of the ovary.

Primary ovarian neoplasms may produce certain morphologic cellular changes and characteristic cytodifferential cellular patterns in peritoneal fluid according to their histogenic background. These may aid in the diagnosis of developing neoplastic ovarian tumors in women years before malignant growth extends beyond the ovary.

TABLE I. *Peritoneal fluid cell count in control mice and in mouse with an ovarian teratoma at age 37 weeks*

	No. mice	Count 250 cells		
		Mesothelial	Lymphocytes	Bare Nuclei
Control	71	110.1 ± 11	93.7 ± 8.0	45.9 ± 10.0
Teratoma	1	80	74	88

±95% confidence limits

SUMMARY

The morphologic cellular and cytodifferential changes in peritoneal fluid accompanying development of spontaneous malignant solid ovarian teratoma in a mouse is reported. Morphologic cell changes appeared in the peritoneal fluid two and one-half months before autopsy of the mouse revealed the unilaterally confined ovarian neoplasm. Their value as possible diagnostic aid for primary ovarian neoplasms in women is emphasized

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Mycoplasma and Vaginal Cytology

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Mycoplasma, earlier named pleuropneumonia-like organisms (PPLO), are the smallest microorganisms known that are capable of growing on a cell-free medium. Lacking a cell wall, they are highly pleomorphic and resistant to penicillin. They are sensitive to several broad-spectrum antibiotics, especially to tetracyclines.[14, 21]

Mycoplasma hominis, *Mycoplasma fermentans* and T-mycoplasmas have been isolated from the genito-urinary tract, and their role in genital inflammatory conditions has been discussed in numerous reports during the last three decades.[7, 19] Although *Mycoplasma* frequently occur in the lower genital tract, only single reports have appeared in the literature where attempts have been made to obtain cytologic evidence for the presence of *Mycoplasma*.[11, 17]

The aim of the present study was to determine if mycoplasma organisms in the lower genital tract of the female produce cytologic changes that could be recognized in Papanicolaou stained vaginal smears. Related to this, the possible influence on the cell morphology of *Trichomonas vaginalis* and *Candida* was also considered.

MATERIAL AND METHODS

The 241 investigated women were divided into four groups according to clinical findings:

Group 1 consisted of 108 non-pregnant, non-puerperal women with clinical signs of genital infection.

Group 2 comprised 14 pregnant women with clinical signs of genital infection.

Group 3 included 81 non-pregnant, non-puerperal women in whom the gynecologic examination revealed normal conditions.

Group 4 was made up of 38 pregnant women with no signs of genital infection.

The clinical criteria for the diagnosis of infection and normal conditions of the genital tract were the same as those reported earlier.[14, 21] The age distribution of the persons investigated is presented in Table I. The women in the different groups are comparable as to marital and socio-economic status. All were sexually active. Almost all the pregnant women were in their first trimester.

Vaginal Smears

Specimens from the endocervix were collected with cotton-tipped swabs. Specimens from the portio and the posterior vaginal fornix were collected with an Ayre's spatula. The specimens were spread onto the same slide. The smears were immediately fixed in 96 per cent ethanol and stained according to Papanicolaou.

The following classification was employed for describing the cellular findings in the smears:

Benign (normal cells)
Benign cell changes (inflammatory atypia)
Slight-moderate dysplasia
Strong dysplasia—suspicion of malignancy

There was no case in the present series in which cytologic findings definitely indicated carcinoma *in situ* or invasive carcinoma.

The methods used for the isolation of *Mycoplasma*, *Neisseria gonorrhoeae* and *Candida* have been described earlier.[11] For the species determination of *Candida*, the methods used by Wallerström[19] were employed.

For the demonstration of *T. vaginalis* the following three methods were used:

a) examination of Papanicolaou stained smears;

b) material from the posterior vaginal fornix was examined as wet smears immediately after sampling;

c) specimens from the posterior vaginal fornix were also cultured in Oxoid Trichomonas medium No. 2 containing 8 per cent horse serum, penicillin 1 000 i.u./ml and streptomycin 500 mg/ml. The specimens were transferred directly after sampling into the medium that before inoculation had been heated to 37°C. Incubation was made at 37°C and the test tubes were examined under a microscope repeatedly for up to two weeks before discharged.

Clinical data concerning infection and the results from the cultures were not known by the cytologists at the time of the examination of the smears.

Treatment and Follow-up

Twenty-two women in Group 1 were treated with 600 mg of metacycline (Rondomycin[4]) orally daily for eight days, and 15 were prescribed 1000 mg of chloramphenicol (Kloramfenikol[5]) in combination with 1200 mg of phenoxymethylpenicillin

(Calciopen®) orally daily for 10 to 14 days. In most of the cases, fungicide (Pimafucin®) was administered locally during the period of antibiotic therapy. The patients in whom *T. vaginalis* had been demonstrated, 600 mg of metronidazol (Flagyl®) was given orally daily for one week in addition to the antibiotic treatment. Repeated examination and collection of specimens for culture and cytologic examination were made within one week after the treatment was concluded. The women were urged not to have sexual intercourse until then.

RESULTS

Cytology Related to Cultural Finding

The cytologic findings in the vaginal smears from the women in the different clinical groups are presented in Table II.

M. hominis was isolated from the genital tract in 58.3 per cent of the women in Group 1, in 64.3 per cent in Group 2, in 4.9 per cent in Group 3 and in 2.6 per cent in Group 4. The isolation rate of *M. hominis* in women presenting various grades of cell changes is shown in Table III. The women with clinical signs of genital infection (Groups 1 and 2) and who harboured *M. hominis* had a benign cytology in 12.5 per cent. In 69.4 per cent there were benign cells with inflammatory changes. Slight-moderate dysplasia was found in 16.7 per cent and severe atypia in 1.4 per cent. The corresponding figures for women in the same clinical groups not harbouring *M. hominis* were 32, 54.8 and 6 per cent. *M. hominis* was recovered significantly less often from the women who had a normal cytology than from those presenting smears with inflammatory changes or slight moderate dysplasia ($\chi^2 = 38.6$ $p < 0.001$).

M. hominis was rarely isolated from women in which the vaginal smears revealed flora dominated by bacteria morphologically of Döderlein's type, and women presenting such smears had generally no clinical signs of genital infection (Table IV).

About 25 per cent of the examined vaginal smears had microscopically characteristic "dirty" appearance. These smears displayed coccoid, basophilic granula—often covering the cells and also occuring in great numbers between them. Between the cells, strands of material stained bluish and gave the smears an impression of having a hairy covering (Fig. 1). As appears from Table V "dirty" vaginal smears were found significantly more often in cases from which *M. hominis* was isolated than in those from which this organism had not been recovered ($\chi^2 = 106.0$, $p < 0.001$).

The smears from 67 of the women investigated contained several squamous epithelial cells of superficial, and sometimes of intermediate, origin showing perinuclear halo. We consider perinuclear halo as circular translucent area

TABLE I *Age Distribution*

Age	Group				Total
15–19	22	1	7	7	37
20–24	61	4	34	7	106
25–29	13	4	15	6	38
30–34	5	4	8	7	24
35–39	4	0	6	7	17
40–44	1	1	4	4	10
>45	2	0	7	0	9
Total	108	14	81	38	241

TABLE II *Percentage Distribution of Cytologic Findings Within the Different Clinical Groups of Women*

Clinical Group	Number of Cases	Smears of Type (Per cent of N)			
		Benign	Benign (Inflammatory Changes)	Slight-Moderate Dysplasia	Strong Dysplasia
1	108	20.4	62.0	13.9	3.7
	14	21.4	71.5	7.1	0
3	81	60.5	37.0	2.5	0
4	38	65.8	31.6	2.6	0

TABLE III *Isolation Rate of M. hominis Correlated to the Cytologic Findings*

Cytologic Classification	Number of Cases	*M. hominis* Isolated	
			Per cent
Benign	99	9	9.1
Benign (inflammatory changes)	119	55	46.2
Slight-moderate dysplasia	19	12	63.2
Strong dysplasia	4	1	

TABLE IV *Döderleins' Bacilli in the Vaginal Smear in Relation to Clinical Grouping and Isolation of M. hominis*

Cytologic assessment of bacterial flora in smears	Total number of cases	Number of cases with clinical signs of infection	*M. hominis* isolated
Döderlein bacillus predominating	119	18	10
Other bacteria predominating	122	104	67

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TABLE VI *Incidence of Smears Containing Cells with "Perinuclear Halo" in Women Harbouring M. hominis and T. vaginalis*

Cells with perinuclear halo	Number of cases (N)	Micro-organism isolated							
		M. hominis but not T. vaginalis		T. vaginalis but not M. hominis		Both M. hominis and T. vaginalis		Neither M. hominis nor T. vaginalis	
			per cent of N		per cent of N		per cent of N		per cent of N
Found	67	24	35.8	4	6.0	17	25.4	22	32.8
Not found	174	31	17.8	5	2.9	5	2.9	133	76.4

TABLE VII *Isolation of M. hominis and Number of Women Presenting "Dirty" Smears and Smears with Predominance of Döderlein's Bacillus Before and After Treatment with Metacycline and Chloramphenicol in Combination with Penicillin*

	Number of Cases	Findings in Cultures or Smears		
		M. hominis Isolated	"Dirty" Vaginal Smears	Döderlein's Bacillus Predominating in Smears
Metacycline				
before treatment	22	13	12	0
after treatment	22	1	2	17
Chloramphenicol and penicillin				
before treatment	15	11	9	3
after treatment	15	8	5	6

isolated from the apparently healthy women with normal flora dominated by Döderlein's bacillus. The isolation rate of *C. albicans* in the groups of pregnant women with and without signs of genital infection (Groups 2 and 4) was significantly higher than in the corresponding groups of non-pregnant women (Groups 1 and 3). *C. albicans* was cultured from 22.2 per cent of the investigated women, while the cytologic examination suggested *Candida* in 5.8 per cent of the cases. The clinical examination revealed an apparent vaginal candidiasis in only five instances.

Observations On The Effect of Antibiotic And Metronidazol Treatment

Twelve of the 22 women given metacycline presented "dirty" vaginal smears before the treatment, but after the therapy "dirty" smears were found in only two cases. Of the 15 women treated with chloramphenicol and penicillin, nine had "dirty" smears before and five after the therapy (Table VII). Thirteen women harboured *M. hominis* before treatment with metacycline had been initiated. After the treatment, the organism was recovered in one instance. The corresponding figure for 11 women treated with chloramphenicol and penicillin was eight.

The smears collected from the women who had taken metacycline showed, in most cases, a bacterial flora dominated by Döderlein's bacillus (Table VII), whereas those women who had been treated with chloramphenicol in combination with penicillin still presented smears with other bacteria predominating.

DISCUSSION

Ultrastructural studies have shown that mycoplasma organisms often attach themselves to the surface of cells in tissue cultures[8] and in vivo to cells of infected tissue.[14] In electron microscope preparations of vaginal discharge from women harbouring *Mycoplasma* these organisms were often seen to be localized on the cell membrane of the vaginal cells. Some workers are convinced that mycoplasma organisms occur intracellularly while others have not been able to confirm the intracellular presence of these organisms. Interference with the function of the cell membrane by mycoplasma organisms has been suggested to result in cell damage. In tissue cultures infected by *Mycoplasma*, cell damage has also been related to depletion of arginine in the cultural medium.

Chromosomal changes in tissue culture cells have been attributed to *Mycoplasma* infections.[17]

In the present series of women with clinical signs of infection, slight or moderate atypia was found numerically more often in the smears of those harbouring *M. hominis* than in the women from whom this organism had not been cultured (87.5 and 68.0 per cent respectively). This finding requires further evaluation.

Jones and Davson found that vaginal smears of women from whom cultures had yielded a heavy growth of *M. hominis* generally had a "dirty" appearance, and that such smears only occasionally were found in women not harbouring this surrounding the nucleus and sharply demarcated by the cytoplasm (Fig. 2). Of all the investigated women harbouring *M. hominis* 48 per cent presented vaginal smears containing cells with a perinuclear halo. Of all women yielding smears with cells with perinuclear halos, 35.8 per cent harboured *M. hominis* but not *T. vaginalis*, 6 per cent *T. vaginalis* but not *M. hominis*, 25.4 per cent both of these organisms, and 32.8 per cent neither of these organisms (Table VI).

Mycoplasma fermentans was isolated from only one of the investigated cases.

T mycoplasmas were, in contrast to *M. hominis*, often recovered from women with cytologically normal smears (41 out of 99) and from women whose smears manifested a flora dominated by bacteria morphologically of Döderlein's type (43 out of 119). No correlation was found between T-mycoplasmas and "dirty" smears, nor between T-mycoplasmas and smears containing cells with a perinuclear halo. The same seemed to be valid for *N. gonorrhoeae* as estimated from the small number of cases (20) in the present material.

FIG. 1. "Dirty" smear with numerous coccoid granula covering the epithelial cells and the spaces between them (× 400).

TABLE V. *Incidence of Dirty Vaginal Smears in Relation to the Recovery of M. hominis*

M. hominis	Number of Cases	"Dirty" Smears	
			Per cent
Isolated	77	51	66
Not isolated	154	7	4.6

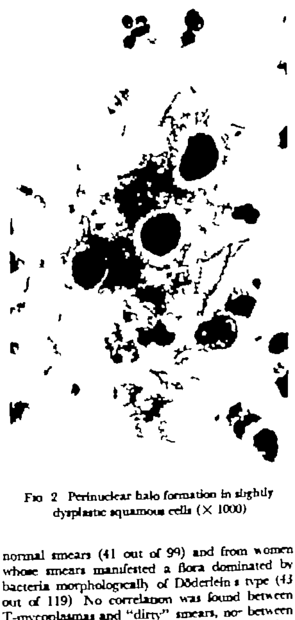

FIG. 2. Perinuclear halo formation in slightly dysplastic squamous cells (× 1000).

There was no difference between the pregnant and the non-pregnant women as regards the frequencies of "dirty" vaginal smears and smears containing cells with a perinuclear halo.

T. vaginalis was demonstrated in 31 of the cases in Group 1 and 2 but in none of the cases in Group 3 and 4. Thus all women harbouring *T. vaginalis* had clinical signs of infection. In the 31 cases *T. vaginalis* was found by the cytologic examination in 21 cases, by microscopy of wet smears in 19 cases. In cultures *T. vaginalis* was found less frequently (15 cases) than with the other two methods.

Candida albicans was found in 15.7, 35.7, 24.7 and 34.2 per cent of the cases in Groups 1, 2, 3 and 4 respectively. Thus *C. albicans* could often be studied in relation to this. Our material comprised 241 cases.

In women presenting vaginal smears that displayed inflammatory atypia or slight-moderate dysplasia, *Mycoplasma hominis* was isolated significantly more often than from those who had smears with normal cytologic findings

Vaginal smears with characteristic dirty" appearance were found significantly more often in women harbouring *Mycoplasma hominis* than from those not harbouring this organism Antibiotic treatment eradicating *M hominis* resulted in normalization of the cytologic pattern N correlation was found between dirty" smears and any of the other micro-organisms studied

Vaginal smears containing epithelial cells with "perinuclear halos were found in the same frequency in women harbouring *M hominis* as in women with trichomonal infestations From all women presenting smears with cells with "perinuclear halos" *M hominis* was found twice as often as *T vaginalis*.

The occurrence of T-mycoplasmas could not be correlated with any of the above-mentioned cytologic findings

A comparison of the effectiveness in detecting *Candida* and *T vaginalis* by clinical, cytologic, and cultural methods is also presented.

ACKNOWLEDGMENT

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organism. In the present investigation the same observation was made and furthermore antibiotic treatment eradicating *M. hominis* generally resulted in a disappearance of the "dirty" impression of the smears.

In the clinically healthy women (Groups 3 and 4) "dirty" smears were found occasionally, namely in two cases and one case, respectively. In two out of these three cases, *M. hominis* was isolated from the lower genital tract. In the women harbouring *M. hominis* and who had clinical signs of infection (Groups 1 and 2) 68 per cent presented "dirty" smears. Among all women in these latter groups with "dirty" smears, 89 per cent harboured *M. hominis*. Hence the results of the present study indicate that a woman with a "dirty" vaginal smear is likely to harbour *M. hominis*.

Not having made cultures for T-mycoplasmas, Jones and Davson[18] left the possibility open that these organisms could be related to the "dirty" appearance of vaginal smears. In our series, no correlation could be demonstrated between "dirty" smears and the occurrence of T-mycoplasmas. Nor could any significant correlation be demonstrated between "dirty" smears and *N. gonorrhoeae*, *T. vaginalis* or *C. albicans*.

Shephard[19] reported the finding of intracytoplasmic inclusions in squamous cells in methanol fixed, Giemsa or azure B-eosin stained smears of urethral scrapings collected from men with non-gonococcal urethritis. Within one cell, these inclusions varied from a few discrete particles to such a great number that they almost completely filled the cytoplasm. Shephard attributed these inclusions to the occurrence of T-mycoplasmas. In the present series of Papanicolaou stained vaginal smears, no obvious correlation could be demonstrated between intracytoplasmic inclusions and the presence of T-mycoplasmas.

The nature and significance of the "perinuclear halo" are obscure. Ayre[4] suggested that "the significant feature of the halo cell would seem to be that it represents a stepping stone between normal cells and malignant or premalignant cells" and that it might be the result of a virus infection of the cells. Virus infections of the lower female genital tract are extremely rare as far as hitherto reported with the exception of *Herpesvirus hominis* infections. In the present series, no cytologic or clinical evidence of a *Herpesvirus hominis* infection was found in any of the cases.

The appearance of cells with a perinuclear halo in vaginal smears has also been related to trichomonal infestations. Koss and Wolinska[12] found such cells in the smears in about 60 per cent of a series of women with trichomonal infestations. These authors suggested the perinuclear halo to be "the cytologic counterpart of the frequently considerable intracellular vacuolization (edema ?) of squamous epithelium noted in Trichomonas cervicitis". In our series, halo cells were present in 68 per cent of the cases in which *T. vaginalis* had been demonstrated, but only half of the women with such smears harboured *T. vaginalis*. Cultures from the majority of women in whom *T. vaginalis* has been demonstrated also yielded growth of *M. hominis*, or in 58 per cent of all women from whom this organism was cultured. From the present series, it appears that neither *T. vaginalis* nor *M. hominis* had any close relationship to the presence of a perinuclear halo.

Gray, Harper and Still[7] reported that one week of treatment with metronidazol was generally sufficient to eradicate cytologic signs of trichomonal infestations and also halo cells. In our series of women with trichomonal infestations, cells with a perinuclear halo were found as often in the smears collected before as after treatment with metronidazol.

Koss *et al.*[11] found in smears from women treated with intravaginal oxytetracycline suppositories or powder a cytologically "clean" appearance. These smears were remarkably free of bacteria, especially Döderlein's bacillus. In the present study, oral metacycline treatment resulted in smears that also gave such a "clean" impression, but there was generally a flora dominated by Döderlein's bacillus. In our opinion a predominance of Döderlein's bacillus should be regarded as the normal state in the vagina of healthy females in the childbearing age.

Owing to the presence of a variegated flora of micro-organisms in infectious conditions in the lower genital tract of the female, the role of a single species is often difficult to assess. However, the results of earlier studies suggest an etiologic role of *M. hominis* in infections of the female genital tract.[11, 20] The possibility that recurrent or long standing infections in the lower genital tract may cause cell abnormalities makes a correct diagnosis and adequate treatment mandatory, as it might be possible not only to cure the infection but also to provide optimal conditions for a normalization of the cellular morphology. In this respect, *Mycoplasma* also has to be taken into consideration.

For a further evaluation of the interrelationship between *Mycoplasma* and the squamous epithelium of the uterine cervix, electron microscopic studies are in progress.

SUMMARY

The aim of the present study was to determine if the occurrence of *Mycoplasma* in the female genital tract produces cytologic changes that can be recognized in Papanicolaou stained vaginal smears. *Candida albicans*, Döderlein's bacillus, *Neisseria gonorrhoeae* and *Trichomonas vaginalis* were also

*

other cells are eosinophilic. Blue cells originating from the fetal mouth and urinary system are found to much lesser degree where a male fetus is concerned.

It appeared that the fetal sex could be estimated with reliability between the 31st and the 37th pregnancy week, if 20 per cent count of blue cells is considered maximum for a male fetus, and 22 per cent as the minimum for female. The round cyanophilic urinary cells, usually present in small numbers, were not included in the count.

I is the purpose of the present investigation, made by an observer who is not trained in cytology (J.H.A.) to have the criteria which were mentioned above tested on new material, to analyze possible sources of errors, and to get an impression as to the reliability of interpretation by several observers.

MATERIAL AND METHOD

One or more samples of amniotic fluid were obtained from 57 pregnant women between the 30th and 37th weeks of gestation. This supplied a total number of 67 smears, which, stained by the Harris-Shorr method, were examined under high power (× 400) without knowledge of the fetal sex. It appeared that from the 67 smears 33 originated from pregnant women with male fetus and 34 from pregnant women with a female fetus. The indication for amniocentesis was Rhesus-sensitization in 51 gravidas, unknown duration of pregnancy in four and premature labor and polyhydramnios in two patients. I each smear 400 cells were observed.

RESULTS

The results are given in Table I. I 66 of the 67 smears, diagnosis was in agreement with the sex as fixed at birth. The smear leading to false diagnosis originated from woman with Rhesus-sensitization who was 31 weeks pregnant. The slide was of poor quality and the percentage of blue cells was just below 20.

COMMENT

The aforesaid confirms the finding of Rosa and Fanard, that relationship exist between the number of vaginal cells in the amniotic fluid and the fetal sex. The theoretical basis of the method seems to be sound, the test is easily practicable and can be done by non-specialized staff. The reliability (98.5% in this material) is acceptable and will probably improve if the readings are made only on slides of good quality.

It is quite obvious that this method will never take the place of that based on the presence or absence of sex-chromatin. It might, however, be an additional safeguard in some cases. In addition, for diagnosing abnormalities in the cytologic features of amniotic fluid caused by congenital anomalies of the fetus, it is necessary to first know the normal pattern of the cytogram, and the difference between male and female staining differentiation.

SUMMARY

Prenatal prediction of fetal sex appears feasible by counting the proportion of fetal vaginal cells in the amniotic fluid between the 32nd and the 37th week of pregnancy.

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A Simple Method for Prenatal Determination of Sex

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To look for sex-chromatin in the nuclei of amniotic fluid cells is a well-known and reliable method of prenatal determination of sex. A disadvantage, however, is that a sufficient number of unimpaired nuclei are not always found in the smear.

In a former study[1] it was stated that with the presence of a female fetus a great number of vaginal cells are present in the amniotic fluid, the cytoplasm of which turns blue with the application of the Harris-Shorr method of staining. Nearly all

TABLE I

Patient	Week of gestation	Percentage of cyanophilic cells	Sex at birth	False
1	33 34 37	51 66 50	f	
	35	52	f	
3	35	17	m	
4	37	20	m	
5	36	44	f	
6	36	62	f	
7	31	4	f	
8	33 36	11 5 12	m	
9	33 35	2 5 12	m	
10	34 36	8 5 11 5	m	
11	31	65	f	
12	34	41	f	
13	35	40 5	f	
14	13 32 33 34 37	13 17 5 16 5 14 18	m	
15	31 32 34	11 7 5 9	m	
16	36	10	m	
17	37	13	m	
18	35	23	f	
19	35	7	m	
20	36 37	23 45 5	f	
1	34	6 5	m	
22	35 37	12 7	m	
3	33 34 37	71 63 56	f	
24	32 34	13 1	m	
25	35 36 37	8 11 16	m	
26	36	13	m	
27	31	60	f	
28	36	7 5	m	
29	35 36 37	70 66 60	f	
30	32 34 36	14 6 5 8	m	
31	31 32 32	59 58 5 44	f	
32	32 34 37	53 5 64 29	f	
33	31 32 33 35 36	50 5 56 49 53 59	f	
34	37	73 5	f	
35	37	15	m	
36	31	19 5	f	
37x	35	38 5	f	

x) Numbers 36 and 37 represent two subsequent pregnancies in the same woman.

Acta 5252 p 1

widely different experience in cytology in terms of prevalence of cervical carcinoma, in terms of means of diagnosis and of treatment and each of us brought his own experence to the Committee work.

The members of the International Reference Center for nomenclature in Cytology were

Dr N Aristizabal, Colombia
Dr W Christopherson, U S A.
Dr O A. N Husain, U.K.
Dr L Koprowska, U.S.A.
Dr U Luthra, India
Dr A Meisels, Canada
Dr M. Nasiell, Sweden
Dr V Nuovo, France
Dr A. C. Petrova, USSR
Dr A. Prado, Chile
Dr J W Reagan, U S.A.
Dr G Riotton, Switzerland
Dr J Siracky Czechoslovakia
Dr H Zinser F R. Germany

The most difficult area of the work has been the "precancerous lesions" of the cervix the dysplasias and carcinoma in situ. It was felt the committee arrived at acceptable working terms for most cytopathologists at this moment. Examples of these include

- Dysplasias
  - mild
  - moderate
  - severe
- Carcinoma in situ
- Carcinoma in situ with minimal invasion
- Occult epidermoid carcinoma
- Invasive epidermoid carcinoma
  - keratinizing
  - large-cell, non-keratinizing
  - small-cell, non-keratinizing

These terms, together with their internationally agreed definitions, will comprise a part of the published nomenclature.

On June 13 1970 the Terminology Committee of the International Academy of Cytology by majority vote approved, recognized and supported the W.H.O nomenclature in cytology with the amended provision that modifications may be made, if needed, in the light of final decisions on the histopathologic nomenclature and classification of uterine tumors. It's hoped that the nomenclature of cytology of the female genital tract which will be published in 1971, and will serve a useful purpose among cytologists throughout the world for communication and for teaching purposes.

The next task of the W H.O International Reference Center for Nomenclature in Cytology will be to formulate and test the nomenclature of cytology of the extra-genital tract.

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W H O Nomenclature in Cytology

The World Health Organization cancer control activities are mainly focused on the problem of clinical cancer from the diagnostic and therapeutic point of view and for that reason, was led to promote and achieve an international standardized nomenclature in pathology, hence cytology. There are now 17 groups working on different tumor sites—five groups have completed their task: lung, breast, soft tissues, odontogenic tumors and oro-pharyngeal tumors. The lung, breast and soft tissues terminologies are already published. These publications are composed of a text containing the internationally agreed nomenclature and color photographs illustrating each term and, in addition, the same illustrations appear in the form of diapositives accompanying the book. These are very useful for teaching purposes. Approximately five thousand sets are distributed, free of charge, to medical schools throughout the world.

One will probably question why so much effort is being made to establish an internationally agreed nomenclature. The reason is simple: without adequate communication progress is hampered; no valuable comparison will be feasible between workers in the same field or between Cancer Registries, for instance. Epidemiologic studies can not be compared, or even made, if people involved call the same lesion with different names or, as it sometimes happens, different lesions with the same name.

For only a well established common nomenclature permits sound communication which, in turn, will permit workable studies to be achieved on the natural history, that is to say, the behavior of these tumors in different circumstances which is of so much interest to us.

Needless to say, a prerequisite is a perfect technical procedure, especially of the way the pathological specimens are worked up. Another prerequisite to any discussion in the field of so-called "precancerous" or early cancer lesions is that the clinician be aware of the fact he must treat all these types of lesions according to the diagnosis: in the case of the uterine cervix, for instance, coagulation, conization, hysterectomy simple or radical, radiotherapy and so on.

It is known that, today, neither cytology nor histology are sufficient to predict accurately the future of many of these lesions. We know too that there may be different types of lesions, looking more or less alike under the microscope, but having a very different evolution. It is for that reason that the terms agreed on must describe a lesion and should not have a prognostic implication, at least until we know more about the natural history of these lesions.

Now it should be emphasized, and one should remember, that a nomenclature is never established forever. It is a tentative, more or less good, list of definitions on which one may classify for some time, permitting data to be compared, but it can be reevaluated from time to time when scientific knowledge changes. So it will never be perfect, until one knows accurately, in every detail, the behavior of a lesion which is not the case today in most of the cancer field. This means that any nomenclature may be subject to criticism.

The members of the W.H.O. International Reference Center were drawn from very widely geographical areas. One can well appreciate that there were difficulties in arriving at a consensus opinion. Each member of the Committee has had a

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NEWS FROM SOCIETIES OF CYTOLOGY

Indian Academy of Cytologists

The Indian Academy of Cytologists has been recently constituted with the following office bearers

Prof. P N Wahi F.I.A.C.	President
Prof. Usha K. Luthra F.I.A.C.	Secretary
Dr S. Tejuja	Treasurer

and is affiliated as National Society with the International Academy of Cytology

Israel Society of Cytology

The current Executive National Committee of the Israel Society of Cytology is as follows

President Dr A Beemer Pathologist in-Chief, Shmoel Harofe Hospital, Beer Yaacov Israel

Secretary Treasurer Dr E Malberger Oral Surgeon, in-charge, Cytology Lab Rambam Gov t Hospital, Haifa, Israel.

Regional Members Dr E. Levy Pathologist-in-Chief, Central Emek Hospital, Afuleh, Israel
Dr I S Levij, Pathologist, Department of Pathology Hadassah and Hebrew University Medical School, Jerusalem, Israel

National Editor Dr H Suprun, Pathologist-in-Chief, Naharya Gov t Hospital, Naharya Israel.

The Society meets regularly every two months at the various hospitals and clinics in the country and has so far been presented with variegated and interesting programs in General Cytology problems pertaining to techniques and more recently confronted with the task of setting up the Central School of Cytotechnology to be sponsored and financed with the generous help of the W H O Regional Office authorities and co-sponsored by the local Haifa Medical School authorities as well as the Ministry of Health of the Government of Israel.

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BOOK REVIEW

Automated Cell Identification and Cell Sorting. George L. Wied and Gunter F. Bahr (Editors). New York, Academic Press, 1970. 403 pp. $19.50.

This volume represents valuable information for all those interested in interdisciplinary aspects of automated cell identification and cell sorting.

A group of outstanding contributors makes this addition to the growing list of books on automated laboratory procedures a valuable reference.

The table of contents encompasses the NCI IBM cell separator, buoyant density separation of cells, electronic cell sorting by volume, the rapid cell spectrophotometer, automatic multiparameter analyzer for cells, computerized cytofluorometric research system, chromatin patterns with the fast-scanning stage of a microspectrophotometer, automated leukocyte recognition, automated analysis of cell images by TICAS, and the information theoretic approach to cell identification by computer.

The authors also provide a critical evaluation of the analytic capabilities of the systems, which are still undergoing changes. One of the shortcomings of this book is the fact that the data presented are two years old and may have been superceded by more recent findings.

The book will be of great interest to workers in clinical medicine, tissue culture, genetics, hematology, clinical cytology, and pathology, and is highly recommended.

Marluce Bibbo, M.D., M.I.A.C.

LECTURE at the respective congresses. The administration of the award is in the hands of the International Academy of Cytology which has a special Awards Committee for this purpose. The awardee receives a diploma, a gold medal and a check in the amount of $1,500 00.

During the past ten years the following individuals have received the MAURICE GOLDBLATT CYTOLOGY AWARDS

For 1961 George L. Wied, M.D F.I.A.C. (check donated to the Academy)
The Blum-Riese Professor of Obstetrics and Gynecology & Professor of Pathology
University of Chicago
Chicago, Illinois, U.S.A.

For 1962 J Paul Pundel, M.D F.I.A.C.
Professeur agrege
Maternite
Luxembourg, Luxembourg

For 1963 Leopold G. Koss, M.D., F.I.A.C.
Pathologist-in-Chief
Sinai Hospital of Baltimore
Professor of Pathology
Jefferson Medical College
Baltimore, Maryland, U S.A.

For 1964 Hans-Werner Boschann M.D F.I.A.C. (check donated to the Academy)
Professor of Obstetrics and Gynecology
Freie Universitäts-Berlin
Berlin, Germany

Jointly with Carlo Sirtori, M.D F.I.A.C. (check donated to the Academy)
Professor Director of Carlo Erba Foundation
Milano, Italy

For 1965 James W Reagan M.D F.I.A.C.
Professor of Pathology
Case Western Reserve University
Cleveland, Ohio, U.S.A.

For 1966 Gunter F Bahr, M.D F.I.A.C.
Head, Biophysics Branch
Armed Forces Institute of Pathology
Clinical Professor of Pathology
Georgetown University
Washington, D C., U.S.A.

For 1967 Emmerich von Haam, M.D F.I.A.C.
Professor of Pathology
Ohio State University
Columbus, Ohio, U.S.A.

For 1968 Murray L. Barr, M.D
Professor of Microscopic Anatomy
University of Western Ontario
London, Ontario, Canada

The New Zealand Society of Cytology

Next Conference and Annual General Meeting will be held here in Christchurch on October 8 & 9th 1971

Office Bearers of New Zealand Society of Cytology for 1971–72
President—Dr N W Fitzgerald
Secretary—Mr Wayne J Bumstead
Treasurer—Dr L L Treadgold
Committee—Dr J J Sullivan
Dr Roalie Sneyd
Mrs. Rani Parker

The International Academy of Cytology Committee on Registration and Licensure

The following cytotechnologists have successfully completed the Registry Examination by the International Academy of Cytology and are entitled to carry the title C.T (IAC)" behind their names

Reg No 1 Hildegard Friedrich, C.T (IAC)
49 Stoeberlstrasse
Muenchen 21 Germany

Reg No. 2. Lilia Arcarraga Climaco C.T (IAC)
Tetuan, Zamoanga City
Philippines

Reg No 3 (Mrs.) Sunhild Barbara Zobel, C.T (IAC)
Department of Pathology
United States Army Hospital
Munich Germany

Reg No 4 Sarjit Kaur C.T (IAC)
Department of Pathology
Institute of Medical Research
Tull Road
Penang, Malaysia

Eleven Years Maurice Goldblatt Cytology Awards

The MAURICE GOLDBLATT CYTOLOGY AWARD was introduced by the sponsor in the year 1961 The Award is given for meritorious work in the field of clinical cytology The MAURICE GOLDBLATT CYTOLOGY AWARD is usually awarded at the time of the International Cytology Congresses for three years. The awardees are invited to give the MAURICE GOLDBLATT AWARD

LECTURE at the respective congresses. The administration of the award is in the hands of the International Academy of Cytology which has a special Awards Committee for this purpose. The awardee receives a diploma, a gold medal and a check in the amount of $1,500 00

During the past ten years the following individuals have received the MAURICE GOLDBLATT CYTOLOGY AWARDS

For 1961	George L. Wied, M.D. F.I.A.C. (check donated to the Academy) The Blum-Riese Professor of Obstetrics and Gynecology & Professor of Pathology University of Chicago Chicago, Illinois, U.S.A.
For 1962	J. Paul Pundel, M.D. F.I.A.C. Professeur agrege Maternite Luxembourg, Luxembourg
For 1963	Leopold G. Koss, M.D. F.I.A.C. Pathologist-in-Chief Sinai Hospital of Baltimore Professor of Pathology Jefferson Medical College Baltimore, Maryland, U.S.A.
For 1964	Hans-Werner Boschann, M.D. F.I.A.C. (check donated to the Academy) Professor of Obstetrics and Gynecology Freie Universität-Berlin Berlin, Germany
Jointly with	Carlo Sirtori, M.D. F.I.A.C. (check donated to the Academy) Professor Director of Carlo Erba Foundation Milano, Italy
For 1965	James W. Reagan, M.D. F.I.A.C. Professor of Pathology Case Western Reserve University Cleveland, Ohio, U.S.A.
For 1966	Gunter F. Bahr, M.D. F.I.A.C. Head, Biophysics Branch Armed Forces Institute of Pathology Clinical Professor of Pathology Georgetown University Washington, D. C., U.S.A.
For 1967	Emmerich von Haam, M.D. F.I.A.C. Professor of Pathology Ohio State University Columbus, Ohio, U.S.A.
For 1968	Murray L. Barr, M.D. Professor of Microscopic Anatomy University of Western Ontario London, Ontario, Canada

Jointly
with **Ewart G Bertram, Ph.D**
Professor of Anatomy
Faculty of Medicine
University of Toronto
Toronto, Ontario, Canada

For 1969 **Paul Lopes-Cardozo, M D F I.A.C.**
Head Department of Hematocytology
Department of Medicine
University of Leyden
Leyden, The Netherlands

Jointly
with **Joseph Zajicek, M D F I.A.C.**
Associate Professor and Director
Department of Cytology
Karolinska Institutet
Stockholm, Sweden

For 1970 **Arthur I Spriggs, M D F I.A.C.**
Director of Cytopathology
Churchill Hospital
Oxford England United Kingdom

For 1971 **Stanley Fletcher Patten Jr. M D F I A C**
Professor and Chairman
Department of Pathology
University of Rochester Medical Center
Rochester New York U S A.

The next three years awards will be presented at the *Fifth International Cytology Congress* Bal Harbor Hotel, Miami, Florida, U S.A (May 29–June 2, 1974) Nominations may be submitted to

The International Academy of Cytology
Maurice Goldblatt Awards Committee
5841 South Maryland Avenue
Chicago Illinois 60637 U.S A

Nominations must be accompanied by (1) a reasoned statement why the award should be given to the nominee, (2) his curriculum vitae, and (3) his list of publications Every Fellow and Member of the International Academy and every Member of the Faculty of any University may submit nominations. Self-nominations are unacceptable In order to be considered for the 1974 presentations, the nominations for the 1972, 1973 and 1974-Awards must be received in the above office not later than July 1 1973

LETTERS TO THE EDITORS

On Barr Bodies and "Cell Senescence"

To the Editors

Drs. George Wilbanks and James Campbell recently reported in this journal on cinematographic observations of Barr bodies and their relation to cell division (In vitro observations of sex chromatin in cells from the human uterine cervix. Acta Cytol 15 297 302, 1971) They conclude with the hypothesis that the Barr body is related to the differentiation of the cell, I It is only present in differentiated cells not in the reproducing, or more undifferentiated cells"

I have for some years looked for Barr bodies in tissue imprints and routine histopathologic material and was impressed by the low frequency of these heterochromatic bodies in highly differentiated tissues I wish to offer an alternative interpretation to Drs Wilbank and Campbell' observations, namely that the Barr body represents sign of cell senescence I further suggest that this state occurs in cell populations regularly undergoing renewal and that the Barr body marks cells with low or lost capacity for division

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Washington D C. 20305 U.S.A.

Effect of 3-Isothiazolone on Human Cancer Cells

To the Editors

This is to report the marked inhibitory effect of 3-Isothiazolone[1] on human cancer cells *in vitro* A concentration of 10 mcg/ml completely inhibited the growth HEp 2 cells in two separate tests conducted on different days with different cell lots.

The tests were conducted by modification of a method previously reported HEp 2 cell sheets in young plastic flask cultures were washed twice with cold versene solution (0 2% in Ca & Mg-free phosphate buffered saline) after removal of the medium. The cultures were then versenated and uniform cell suspension prepared by gently forcing the cells in and out of 10 ml pipette After centrifugation at low speed, the cells were resuspended in Eagle[2] Basal Medium (Hanks salts) containing 10% fetal bovine serum, transferred to stoppered bottle and stirred slowly at room temperature for 15 to 30 minutes by means of magnetic stirrer The concentration of living cells was determined by differential counts in hemocytometer after staining with 0 5% trypan blue aqueous solution.

The compound was completely soluble in the tissue culture medium and serial dilutions were prepared in cold medium from 10 mg/ml stock solution just before testing Drug-cell test suspensions were prepared by adding 0.5 ml of the drug dilutions to 4 5 ml portions of the HEp 2 cell suspension Tube cultures, 3 to 5 per drug dilution, were prepared by adding 1 ml portions of the drug-cell test suspensions to plastic screw cap tissue culture tubes. The cell control cultures were prepared by adding 0.5 ml of medium instead of drug dilution to 4 5 ml of the cell suspension. The cultures were incubated at 36°C and observed for a period of seven days The inoculum of HEp 2 cells in the first test was 81 000/ml and 155,000/ml in the second test 3-Isothiazolone at concentration of 10 mcg/ml completely inhibited growth of the cancer cells in both tests A concentration of 1 mcg/ml was without effect. A number of other drugs tested had no inhibitory effect on the HEp 2 cells

Chromosome studies revealed that in spite of its marked anti-mitotic effect, 3-Isothiazolone does not cause chromosome abnormalities Evaluation of this drug against transplantable human tumors in experimental animals is planned.

Donald W McKinstry Ph.D
William L. West, Ph D
Sidney Green, M S
Wilfred D Crow Ph D ** and
Nelson J Leonard, Ph D
College of Medicine Howard
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and Chemical Engineering University of Illinois, Urbana,
Illinois

Present address Department of Chemistry The Australian National University Canberra City Australia

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PRESIDENTIAL ADDRESS

Robert H Fennell, M.D

Department of Pathology Presbyterian-University Hospital of the University Health Center of Pittsburgh, Pittsburgh, Pennsylvania 15213

Presidential addresses in the past have served as platform to deal with some of the broad problems we are confronted with in Cytology Continuing in this tradition, I would like to present you with some thoughts about what I think constitutes good cytology laboratory

The first question is what role this Society should play in upholding adequate laboratory standards Before I attempt to answer this question, let me emphasize that we, as an organization, do not have power to control and regulate standards for the practice of Cytology Due to historical happenstance actual control of the practice of medicine and allied areas is under the individual state jurisdiction These local legislative bodies have enacted rules and regulations to govern our conduct For example As physician, I cannot practice medicine unless I have state license Regulation of laboratories is also often done by the state and sometimes by county or other administrative units within the state The Federal Government, in the past, has participated very little This is rapidly changing, however and it behooves us to inform those who legislate and decree of our ideas about laboratory standards Accordingly I wish to address myself to this

Our Constitution speaks very clearly to this matter Article II, Section VII cites the problems of personnel and their qualifications Section X, however reads as follow

> To encourage the maintenance of adequate standards for early cancer detection, diagnosis, teaching training, and research by providing an authorized stamp of approval for all acceptable Cytology Centers

With this as our guide-line, we should be prepared to give recommendations concerning laboratory standards to the various organizations equipped with the legal authority to approve or more important, disapprove the operation of cytology laboratories Organizations such as the American Society of Clinical Pathologists, (ASCP) and College of American Pathology (CAP) have, the experience and personnel in actual performance of inspection of laboratories.

Who should be working in the cytology laboratory? Undoubtedly the person examining and making decisions concerning cell spreads should be registered cytotechnologist with the necessary training this title implies The director of the laboratory should be someone interested in, and experienced in Cytology and directly available In most situations, this is the Pathologist. However the fact that this person is certified in Pathology does not indicate, necessarily that he has special interest and skills as cytopathologist

The laboratory as part of the practitioner office, should not be exempt from inspection, as it is in some states Private physicians do employ high school graduates, provide few weeks of supervised work, and then regard these persons as laboratory technicians If the regulations do not involve the physician private office laboratory poor quality may also invol Cytology

What quantity of work should laboratory have to be an approved laboratory? We have often thought that 5,000 smears per year was an acceptable volume for single technician I good laboratory one should approach this volume, but I know that good work can be done with lighter load We should not be too rigid, but certain minimum and maximum volume is desirable, which can be altered to comply to the conditions that prevail locally

The good cytology laboratory reports the result of the examination so as to give the patient physician maximum information This should include prediction of the histologic findings. Numbers, therefore, as method of communication, are not acceptable If the preparation is unsatisfactory then it should be so indicated Standards for the adequacy of smear should be high because the cytopathologist only harms the patient in trying to interpret thick, inadequate or poorly fixed preparation Of course the most dangerous result of inadequate studies is false sense of security

Every cytology laboratory should have file of patient names with their diagnoses. Cross indexing should be done so that review of interesting or problem material is available It is important that we look back at classic examples periodically as standards. Follow-up of individual patients is the most important part of quality control because the biologic behavior of certain lesions is the best verification of our diagnosis and may thus serve as guide in deciding on future cases.

Quality control is complex problem We may divide the solution into four categories The most simple method is to review a certain percentage of all preparations and have various echelons in the laboratory involved Secondly it is important that old material be reviewed in the light of answers obtained from the clinician who cares for the patient A third and parallel procedure is the review of biopsy or autopsy material Histopathologic review is essential for the good cytotechnologist and it must be done in conjunction with the Pathologist A fourth and most difficult aspect is to determine what is happening to a disease process in the community Certain projects which have been in progress for a number of years are good examples of the epidemiologic approach Both Dr William Christopherson in Louisville Kentucky and Dr Cyrus C. Erickson in Memphis, Tennessee, with their studies have demonstrated that Cytology appears to contribute to diminished prevalence of cervical cancer

Where should the good cytology laboratory be located? Are the laboratories, far removed from the patient and attending physician good cytology laboratories? Certainly they can be and have close liaison with the physician and patient, but as I intimated above, the best Cytology is done by the laboratory located in the community where the cytopathologist is in close contact with the patient s physician.

Some type of refresher course must be a part of good cytology laboratories. Perhaps that is one more area where the American Society of Cytology can continue to play an important role.

The American Society of Cytology therefore should assume a role as authorized by its constitution to establish standards for the practice of Cytology The inspection and certification should be performed by other agencies such as the ASCP and CAP which are equipped to perform inspections, and they must act with authorization from appropriate local agencies. It must be emphasized that good Cytology can be done only by competent cytotechnologists and cytopathologists who are aware of the clinical setting and role of Cytology This, plus good training and constant renewal of knowledge, more than any other one criterion or set of standards, assures high quality performance

Comparison of Hormonal Colpocytology with Endometrial Histology in Gynecologic Patients

J Victor Reyniak, M.D Alexander Sedlis, M.D Dorothy Stone, Ed.D and Pounopen Rimdusit M.D

Department of Obstetrics and Gynecology New York Medical College-Metropolitan Hospital Center New York, New York 10029 U.S.A.

Hormonal colpocytology and endometrial biopsy have been long established as valuable methods for the assessment of the hormonal status of gynecologic patients The normal patterns of vaginal cytology during the menstrual cycle,[1-4] as well as in gynecologic endocrinopathies, have been thoroughly studied and described. In our study the accuracy of colpohormonal interpretation was compared with the diagnostic interpretation of the endometrial histology on biopsy

MATERIAL AND METHOD

Sixty-one patients, undergoing diagnostic studies in the Gynecologic Endocrine and Sterility Clinics at the New York Medical College Metropolitan Hospital Center were included in the study A vaginal smear taken from the middle third of the lateral vaginal wall, and an endometrial biopsy were obtained from each patient during the same examination The smear was air dried, stained with the standard Papanicolaou technique and interpreted by a cytologist using the generally accepted parameters of Maturation Index and the degree of folding and clumping of cells. The endometrial biopsy specimen was obtained with Novak's curette, fixed and stained with hematoxylin and eosin. The histology was interpreted by a Gynecologic Pathologist, according to the criteria of Noyes, Hertig and Rock as modified by Sedlis (Table I).

In 15 patients judged to be in the post-ovulatory phase of the cycle an estimate of exact day was made from the smear alone. While the same attempt was made on the proliferative phase since there was no way to match this with the endometrium these results are not included.

RESULTS

Six biopsy specimens were unsuitable for interpretation. insufficient material, 2 specimen obtained from lower uterine segment, 3 endometrial polyps, 1 the overall unsuitability was 9 5 per cent.

Five smears were unsatisfactory for hormonal evaluation insufficient cellular material or poor preservation, 3 severe vaginitis, 2. The overall rate was 8.1 per cent.

In the 50 sets of slides which remained, the histologic diagnosis of proliferative endometrium correlated with the colpohormonal findings of estrogen effect in 83.6 per cent. Secretory endometrium and post-ovulatory smear correlations were found in 88 0 per cent. (Table II) Discrepancies were found in 3 cases where the cytologic diagnoses of estrogen effect were found with secretory endometrium and in 4 where the cytology was progestational and the endometrium was proliferative. One smear correctly read as atrophic proved to be the diagnostic clue on early and unsuspected missed abortions confirmed by the biopsy (Fig 1) Examples of positive and negative correlations of both methods are illustrated in Figures 2 to 5.

In our study group, even in the presence of fairly constant cycle lengths, in the post-ovulatory phase, the stated cycle day corresponded with the histologic date in only 36 per cent of the patients. In 48 per cent the histology lag was two or more days from the stated cycle day and in 16 per cent the histology was more advanced than the history (Table III) Of the 16 smears selected for precise dating 40 per cent coincided exactly

DISCUSSION

Colpocytology has been acknowledged as very valuable method for the assessment of hormonal status of patients in gynecologic practice. The main advantage of the method are ease of performance, lack of discomfort to the patient, feasibility of frequent repetitions, rapidity of diagnosis, and the relative ease of recognition of basic cytologic patterns. However since the vaginal mucosa is a target organ for other than ovarian steroids, both exogenous and endogenous, it can on occasion produce bizarre patterns of cytology difficult to interpret

The estrogenic smear while most easily recognized, can be altered in the presence of non-specific stimuli and factors which may "imitate, modify obscure or interfere with estrogen effect."[7-9] Specific or non-specific vaginal infections, vitamin deficiencies, metabolic disturbances and genetic factors can all alter the target organ response.

Progestational smears are basically more diffi-

TABLE I

Morphologic Changes in the Endometrial Cycle

	Menstrual			Proliferative			Secretory											
	Early	Mid	Late	5–9	10–11	12–14	16	17	18	19	20	21	22	23	24	25	26	27
Surface epithelium	Shed	Shed	Shed	Regenerating	Recovered	Intact												
Glands																		
Shape	T	ST	ST	ST	ST	T	T	T	T	T	T	T	T	T	T	Serrated	S	S
Width		C	C	N	N	N	N	D	D	D	D	D	D	D	D	D	D	D
Secretion	0	0	0	0	0	0	0+	Scanty	+	++	Impacted	Impacted	→					
Pseudostratification	0	0	0	+	++	++	++	0	0	0	0	0	0	0	0	0	0	0
Vacuoles	0	0	0	0	0	0	Subnuclear	Subnuclear	Subnuclear & supranuclear	Subnuclear irregular	0	0	0	0	0	0	0	0
Mitotic figures	0	0	0	+	++	+	+	+	0	0	0	0	0	0	0	0	0	0
Cell height of glands	CL	L	L	L	Med	Med	Med	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL	TL
Stroma																		
Predecidua	++	+	+0	0	0	0	0	0	0	0	0	0	0	0	+	++	Well developed ++	++
Prominence of spiral arterioles	+	0	0	0	0	0	0	0	0	0	0	0	+	++	++	++	++	++
Mitoses	0	0	0	+	+	+	+	+	0	0	0	0	0	0	+	+	+	+
Edema	0	0	0	+	+	0	0	0	0	0	0	+	++	+	+0	+0	0	0
Polymorphonuclear infiltration	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	+	++	++
Necrosis	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hemorrhage	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Note	Cellular debris		Dark stroma light glands								Secretion exhausted glands							

T—Tortuous TL—Tall SC—Simple Columnar ST—Straight D—Dilated L—Low CL—Columnar O—Collapsed N—Narrow and S—Serrated.

cult to interpret since the luteal phase is a multihormone condition and the quantitative measurement of progesterone effect may be impossible. In addition, the cytologic effects of progesterone are not specific and may be imitated by fluctuations in pre-existing estrogen levels or androgenic influences.

The endometrium is characterized by more predictable, multiple and well defined responses to the flow of hormones than the vagina in which maturation, clumping and folding are the primary responses. Thus endometrial changes induced by the presence of progesterone allow for accurate dating of the cycle and such cyclic changes reflect the quality and duration of action of the circulating hormones. This makes endometrial biopsy an unsurpassed method for the appraisal of ovulation and corpus luteum function. It is also more economical and frequently more accurate than biochemical methods involving the recovery of progesterone metabolites.

Endometrial biopsy is not without its disadvantages. The discomfort to the patient is of prime concern and makes repetition of this procedure undesirable. In problems of infertility there exists the possibility of interference with early gestation. It has been suggested that endometrial biopsy might have a therapeutic value where infertility is caused by poor luteal function, the implication being that local irritation may promote increased vascularization and decidual reaction and thus support the maintenance of an implanted ovum.

The presented findings reaffirm the value of colpohormonal examination in the gynecologic practice. The percentage of specimens unsuitable for

TABLE II. *Congruency Between Endometrial Histology and Colpocytology*

Endometrial Histology	Colpocytology Estrogenic	 Progestational	% Congruency
Proliferative phase	19	4	82.6
Secretory phase	3	22	88.0

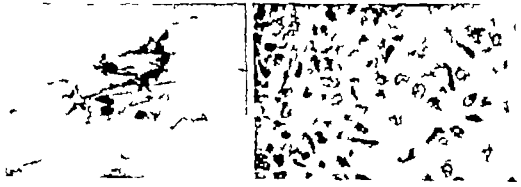

FIG. 1. Colpohormonal and histologic diagnoses: positive correlation; cycle day by history 14; colpohormonal diagnosis: atrophy; histologic diagnosis: decidua. Original Magnification × 368.

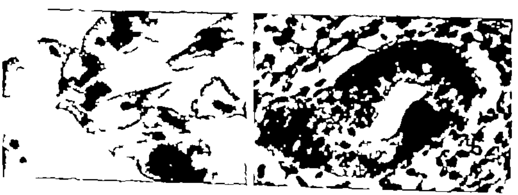

FIG. 2. Negative correlation; cycle day by history 26; colpohormonal diagnosis: moderate progesterone effect; histologic diagnosis: proliferative endometrium. Original Magnification × 368.

interpretation were essentially the same for both methods appraised. In the assessment of cycle phase, the overall congruency of both methods was found satisfactory and correlated well with findings of other authors who, on large series of patients, found such correlation could reach a level of 91 5 per cent.[3] In our series, since the presented material was collected principally from women who were screened on the Gyn-Endocrine and Sterility Service, the discrepancies in the interpretation might be reconciled to cyclic disturbances in these patients. They are also possibly explained by the

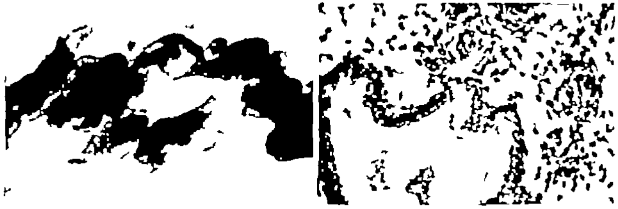

FIG 3 Positive correlation cycle day by history 24 colpohormonal date day 24 histologic date day 23. Original Magnification cytology × 388 histology × 190.

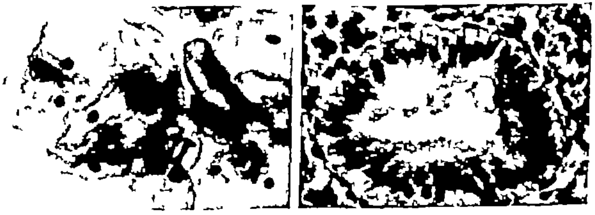

FIG 4 Positive correlation cycle day by history 23 colpohormonal date day 18, histologic date day 18 Original Magnification × 388.

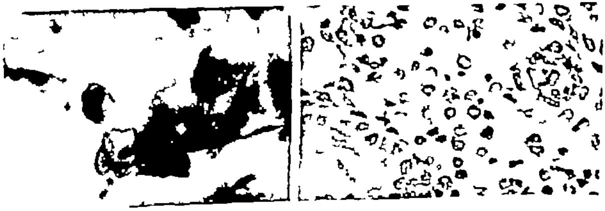

FIG. 5. Positive correlation cycle day by history 26, colpohormonal date day 25 histologic date day 25 Original Magnification × 388

TABLE III. *Correlation Between Cycle Day by History and Histological Dating of the Luteal Phase*

100% Correlation	Histological lag > days	Histology more advanced >2 days
36%	48%	16%

presence of aluteal cycles or insufficient corpus luteum activity. Since the vagina responds to very low levels of steroids it could reflect progesterone in amounts inadequate for the production of endometrial changes. This is particularly true in the early post-ovulatory phase when the vagina, responding more rapidly than the endometrium, makes the colpocytology method more accurate. Wide fluctuation in estrogen levels should also be considered as possible cause of disparity.

It is noted that the correlation between the two methods was somewhat better in the diagnosis of ovulatory cycles. Failures in cytologic diagnosis were most often associated with altered vaginal milieu, such as sub-clinical inflammatory or infectious changes, poor hygiene or frequent douching.

The failures in the interpretation of the endometrial biopsy were mainly attributable to faulty technique. Tissue from the lower uterine segment is unsuitable for hormonal interpretation as is any specimen in which associated pathology is found.

In general, there was good correlation between the two diagnostic methods which reaffirms the use of hormonal colpocytology as an excellent diagnostic office procedure. The practical implication of this study lies in the fact that once the correlation is established for any given patient, the colpohormonal examination can be used with confidence in any follow up required and frequent repeated biopsies can be eliminated.

SUMMARY

The accuracy of colpohormonal interpretation was compared with the diagnostic interpretation of the endometrial histology in 61 patients, in whom both diagnostic procedures were performed during the same examination. Nine point five per cent (9.5%) of smears and 8.1 per cent of biopsy specimens were found unsuitable for interpretation, the reason for the former being usually altered vaginal milieu, and for the latter faulty technique or the presence of unsuspected endometrial pathology.

In the remaining 50 sets of slides, the histologic diagnosis of proliferative endometrium correlated with the colpohormonal findings of estrogen effect in 83.6 per cent. Positive correlations of secretory endometrium and post ovulatory smear were found in 88 per cent. The discrepancies in interpretation could be attributed to disturbances of the cycles in patients included in the study who were women screened in Gyn-Endocrine and Sterility Clinics. This could be further substantiated by observation, that in post ovulatory phase of the cycle, the cycle date by history corresponded with the histologic date in only 36 per cent of patients, while in 48 per cent the histologic lag was 2 or more days and in 16 per cent the histology was more advanced than the history. Correct dating of the luteal phase on the basis of the smear alone was confirmed by histologic diagnosis in 40 per cent of the cases. The overall congruency of both methods in assessment of cycle phase was found satisfactory.

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Clinicopathologic Studies in Feminizing Tumors of the Ovary
III The Role of Genital Cytology

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The ability of ovarian granulosa cell and theca cell neoplasms to synthesize estrogen-like compounds is a characteristic almost unique to them, so rare is it in other tumors. Particularly in childhood and postmenopausal women the action of these substances on other tissues may produce signs and symptoms suggestive of a feminizing tumor long before any mass is demonstrable

A high level of maturation of vaginal squamous epithelium as seen in the vaginal pool smear or lateral vaginal wall scrape is frequently cited as a change of help in the diagnosis of these tumors. The few published studies base their conclusions either upon personal experiences of the reporters with isolated individual cases or with extremely small numbers of cases Schüller[11] reported unusually high Karyopyknotic Indices in 7 feminizing tumors of women between 56 and 70 years of age. Gandefroy reported similar findings in one 66 year old woman. Augustine and Horsky[1] found an "estrogenic" smear in one of four postmenopausal women with granulosa-theca cell tumors de Brux[3] and Rakoff[9] have cited the usefulness of the vaginal smear in these tumors

During the course of our recent review of all available granulosa cell tumors and thecomas in the case records of Duke Hospital, the scarcity of reported documented cytohormonal studies on such patients prompted us to include all available smears in our studies. This paper reports the cytohormonal findings in 12 postmenopausal women with granulosa cell tumors and in 9 postmenopausal women with thecomas.

MATERIALS AND METHODS

In the medical records of Duke University Medical Center dating from its opening in 1930 there are 41 patients with granulosa cell tumors and 20 with thecomas of the ovary

Clinical and Pathologic Findings

A detailed analysis of the clinical and pathologic findings in these patients is under preparation for publication.[6, 7] Some of the more outstanding features are summarized here. On all patients included in this study the surgical pathology reports, operative notes and histologic sections were reviewed in order to verify the presence of granulosa cell tumor or thecoma. The granulosa cell tumors ranged in size from 0 5 cm to 30 cm Twenty-five were greater than 10 cm in diameter and 15 were less than 10 cm. Nineteen tumors were completely solid, 13 were predominantly solid, 6 were completely cystic, and 3 were predominantly cystic. Cut surfaces varied from yellow-white to dark brown in color A tumor was considered to be of granulosa cell type in the presence of a recognizable component of granulosa cells A varied microscopic picture was encountered which included follicular trabecular sarcomatoid and mixed patterns of granulosa cell components Most of the tumors were a mixture of these three patterns.

The thecomas ranged in size from 2.5 to 22 cm Ten of these were 10 cm or greater in diameter Fifteen of the tumors were completely solid five were cystic Cut surfaces were almost invariably described as being grey-white or yellow-white in color The thecomas were composed of plump spindle-shaped uniform cells with round to ovoid nuclei In 16 of the cases, oil red O-stains for fat were available for review and all were positive Thecomas were so-called only in the complete absence of any identifiable granulosa cell component All available sections of endometrium were reviewed in these patients The diagnostic interpretation of these is charted in Tables I and II

In contrast to the current trend in many pathology laboratories to combine granulosa cell tumors and thecomas into the so-called "granulosa theca cell tumor" group we retained a separation of these tumors. Our justification for this is to be found mainly in their clinical behavior Table III shows some clinical observations on patients har

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Dr Goldston's present address is 703 S Clark Avenue Tampa Fla. 33609 boring these tumors. The two groups were similar with respect to the frequency of postmenopausal bleeding and palpable tumor. The thecoma patients were slightly older and more likely to be postmenopausal. The most significant contrast, however, is in mortality from the tumors. Of the 41 granulosa cell tumor patients in the series, 8 (20%) died of generalized metastatic granulosa cell carcinoma. Six of these died within 5 years of diagnosis; the remaining 2 died at 6 and 20 years respectively. Of the patients with thecoma, only one (5%) died of the tumor.

Cytopathology

Cytohormonal evaluation was restricted to the postmenopausal tumor patients because of the in-

TABLE I. *Cytologic and Histologic Findings in Twelve Granulosa Cell Tumors*

Patient		No. yrs PM at DX of tumor	Vaginal pool smear		Endometrium	Comment
Number	Age		Pre-op M I	Post-op M I		
C17	77	47	0/78/22	100/0/0	not available for study	PM bleeding endometrial cell on pre-op smear
C31	50	10	0/16/84	7/78/15	cystic hyperplasia with polyp	PM bleeding
A32	50	2	0/10/90	0/94/6	cystic hyperplasia	PM bleeding
G62	69	20	0/76/24	0/71/29	cystic hyperplasia	PM bleeding persistent tumor after surgery
97	58	6	0/12/88	74/14/12	cystic hyperplasia	PM bleeding
D21	63	17*	0/78/24	not available	not available	lost to follow-up immediately following surgery
G37	42	6	0/16/84	0/44/56	cystic hyperplasia with polyp	PM bleeding persistent tumor after surgery
E93	56	7	0/66/34	1/80/19	typical hyperplasia	
H37	78	42*	0/13/87	58/26/16	not available for study	
A60	49	8	0/48/52	0/100/0	cystic hyperplasia	PM bleeding
H62	52	4	0/60/40	29/57/14	cystic hyperplasia	PM bleeding endometrial cells on pre-op smear
H58	52	3	0/14/86	0/86/14	proliferative	

* Surgical menopause

TABLE II. *Cytologic and histologic findings in nine thecomas*

Patient		No. yrs PM at DX of tumor	Vaginal pool smear		Endometrium	Comment
Number	Age		Pre-op M I	Post-op M I		
A73	50	8	0/100/0	2/81/17	cystic hyperplasia	PM Bleeding
A04	56	20	0/100/0	0/100/0	cystic hyperplasia	
D54	48	½	0/98/2	0/97/3	cystic hyperplasia	
C75	53	4	0/53/47	91/9/0	cystic hyperplasia	PM Bleeding
E20	55	10*	0/100/0	0/100/0	not available for study	
G94	52	3	0/98/2	5/83/12	inadequate for evaluation	PM Bleeding
G20	60	11	96/4/0	98/2/0	cystic hyperplasia	
H61	79	28	0/47/53	0/90/10	adenocarcinoma	PM Bleeding
G40	54	18	0/17/83	0/23/77	not available for study	

TABLE III *Some Clinical Observations on Granulosa Cell Tumors and Thecomas*

Clinical finding	GCT (41 patient)	Thecomas (20 patients)
Mean age	52.7 years	57 2 years
Postmenopausal	28 patients (83 4%)	15 patients (75%)
PM bleeding	14 patients (53 9% of all PM patients)	8 patients (53 3% of all PM patients)
Abdominal mass	35 patients (85 4%)	20 patients (100%)
Died from tumor	8 patients (19 5%)	1 patient (5%)

creased probability of detecting abnormal estrogen stimulation in this group No cytologic material was available from children with feminizing tumors. As the first cytology laboratory did not open until 1947 only the patients with tumors since that time could be included, a total of 28 granulosa cell tumors and 18 thecomas Eighteen of these 28 granulosa cell tumor patients were postmenopausal. Vaginal pool smears were available for study in 12. Of the 18 thecoma patients, 17 were postmenopausal. Nine of these had vaginal pool smears available for study

As our main interest in the smears centered around evidence of hormonal activity only vaginal pool smears were reviewed Cytologic studies consisted of the following 1) quality of stain and mounting was evaluated. Many of these smears were 20 years old or older When necessary they were decolorized re-stained by the Papanicolaou method, and re-mounted. These procedures in no way disturbed the morphology or relationship of the cells. 2) Each smear was completely rescreened with particular search being made for any cell of possible endometrial origin 3) Cytohormonal evaluation consisted of observation of any interfering factors such as severe inflammation or trichomonas infestation and a differential count of squamous cells For the latter 100 cells were counted in at least 5 widely randomized fields, and from these counts Maturation Indices were computed. A squamous cell was considered to be a superficial cell when its nucleus measured less than 6 microns in diameter and had no discernible chromatin pattern The Maturation Index was computed as a ratio of the percentage of parabasal, intermediate, and superficial cells All screening and counts were performed by one of the authors without prior knowledge of patient status Approximately 140 vaginal pool smears were reviewed

RESULTS AND DISCUSSION

The pertinent cytologic and histologic findings on endometrium in the 12 granulosa cell tumors are summarized in Table I As may be seen from patient age ranged from 42 to 78 years and the last menstrual period had varied from 2 years prior to diagnosis of tumor to 47 years before. The preoperative Maturation Index is from a smear taken several days before surgery The postoperative smears were taken from 6 weeks to 4 years following surgery All of the 12 patients exhibited a preoperative maturation of the squamous epithelium which was inconsistent with their age and menstrual status. In all of these the Maturation Index showed a moderate to marked shift to the right with 6 patients having a superficial cell component of 80 or greater The smears also showed clean backgrounds, flattened squamous cells lying singly and the presence of keratohyaline granules in many of the superficial cells. Following surgery in 8 patients the first available smear showed a superficial cell component of 19 or less. In 6 of these it was 15 or less at a 2 year postoperative check. In 2 patients abnormal cytohormonal patterns persisted after surgery Patient G62 with a postoperative Maturation Index of 0/71/29 was known to have persistent tumor Patient G37 with a postoperative Maturation Index of 0/44/56 was known to have, not only residual granulosa cell tumor but also adenocarcinoma of the endocervix which had been incompletely removed. No postoperative smear is available on Patient D23. She could never be induced to return for check after her surgery Endometrial cells were present in the preoperative smears of two of the patients Endometrium was available for study in 9 of the 12 patients. In 7 patients there was cystic hyperplasia, in one an atypical hyperplasia, and in one a proliferative change similar to that seen within the first fourteen days of the normal menstrual cycle. The endometrial changes are in keeping with the history of uterine bleeding of which 8 of the 12 patients complained.

The pertinent cytologic and histologic findings in the 9 thecomas are summarized in Table II Patient age ranged from 48 years to 79 years and the last menstrual period from 4 months prior to diagnosis of tumor in the 48 year old female to 28 years prior in the 79 year old. The preoperative Maturation Index was from a smear which was taken from the patient varying from 1 day to 3 months prior to surgery In 8 of the 9 patients the smears had been taken at 3 weeks or less before surgery Postoperative smears were taken from 1 day to 9 years following surgery Three of the patients exhibited preoperative Maturation Indices which were inconsistent with their age and menstrual status The remaining 6 showed Maturation Indices which were either compatible with an intermediate cell pattern or atrophic pattern Patient C75 had a smear one week prior to operation which had showed a Maturation Index of 0/53/47 Nine months following operation the smear had reverted to a completely atrophic pattern with Maturation Index of 91/9/0. Patient H61 1 day prior to surgery had had Maturation Index of 0/47/53 Nine months after surgery her smear exhibited mid-zonal shift with Maturation Index of 0/90/10 Patient G40 one week prior to surgery had a Maturation Index of 0/17/83. Two months following operation the Index was persistently abnormal with count of 0/23/77 Although additional smears are not available on this patient, it is known that she is living and apparently well 5 years following surgery Endometrium was available for histopathologic examination in 7 patients I one of these, Patient G94, it was too scanty to evaluate. There was cystic hyperplasia of endometrium in 5 patients and adenocarcinoma of the endometrium in one Four of the 9 had first presented with the chief complaint of postmenopausal bleeding

Multiple studies have confirmed the frequency with which the vaginal epithelium in healthy postmenopausal women may reflect some estrogen-like effect Masukawa studied the hormonal status of 262 postmenopausal women selected from group of 2,103 patients I postmenopausal years 1 to 10, the mean Maturation Index computed as /78/15 in the years 11 to 20 t was 18/75/7 in the years 21 to 30 it was 19/73/8 after 31 postmenopausal years it was 24/66/10 Randall reported an estrogenic effect in 55.2 percent of women of one or more years postmenopausal Smolka and Soost[18] classified 12 per cent of women more than 5 years postmenopausal as having "high proliferation pattern In study of 226 patients at least 2 years postmenopausal, Stone and co-workers[22] found high maturation values in 8.9 per cent of women Frost[4] has reported slightly abnormal cytohormonal patterns in postmenopausal women with shifts to the right associated with cystic and solid tumors of the ovary endometrial hyperplasia and adenocarcinoma, metastatic tumors to the ovary tumors of the tube, tumors of the adrenal, and tumors of the breasts I his studies, superficial cell component of more than 10 was considered abnormal Of those patients exhibiting slightly abnormal Maturation Index shifts to the right, 25 per cent appeared to harbor no disease Thus every cytopathology laboratory doing cytohormonal evaluation must consider the reported levels of epithelial proliferation in postmenopausal women and arrive at some qualitative and quantitative interpretation such that an upper limit of normalcy can be established In our laboratory superficial cell component of 15 is the upper limit of normal Any degree of epithelial proliferation higher than this is reported as abnormal, and the patient is evaluated for possible cause

In contrast to these normal values, or slightly abnormal values in the postmenopausal woman, this study has shown that markedly abnormal cytohormonal patterns occurred in more than 70 per cent of postmenopausal women harboring feminizing tumors of the ovary 1 60 per cent of these abnormal patterns, the superficial cell component was 50 or greater In 2 patients the persistence of an abnormally high superficial cell component after therapy was associated with tumor recurrence It has not been the object of this study to advocate the use of the vaginal smear as primary diagnostic tool for the detection of feminizing tumors of the ovary Rather the purpose has been to emphasize its role as an extremely useful aid in conjunction with history physical findings, and other laboratory studies More than 60 per cent of feminizing tumors of the ovary occur in postmenopausal women or in childhood. It is within these same two age groups that abnormal estrogen stimulation of the vaginal mucosa is most easily discernable.

SUMMARY

This is retrospective study of approximately 140 vaginal pool aspiration smears in 12 patients with granulosa cell tumor and in 9 patients with thecoma These 21 patients represent the total number of postmenopausal women in the Duke University Medical Center series of granulosa cell tumors and thecomas since the opening of the first cytology laboratory in 1947 For the purposes of general cytologic and cytohormonal evaluation, the smears were restained when necessary and rescreened Cytohormonal evaluation consisted of a qualitative statement of suitability of smear and Maturation Index In the immediate preoperative period all 1 of the patients with granulosa cell tumors showed Maturation Indices of 0/78/22, or higher with 6 showing a superficial cell component of 80 or greater Two of the patients had endometrial cells in the smears. At 6 weeks to 8 months following surgery the Maturation Indices had shifted to the midzone or completely to the left in 8 patients I 2 patients Maturation Indices of 0/71/29 and 0/44/56 persisted. These 2 patients were shown to have residual tumor Two patients were lost to follow up For the thecoma group, preoperatively 3 patients showed superficial cell component of 47 or greater The remaining 6 exhibited either a midzonal shift or complete atrophy Following surgery the Maturation Index reverted to midzonal or completely left shift in 2 and remained unchanged in 7 Fiv of these 7 patients showed endometrial hyperplasia.

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Exfoliative Cytology of Primary and Metastatic Wilms Tumors

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Wilms tumor constitutes about 6 per cent of all renal cancers and is the most common renal tumor in childhood It accounts for approximately 30 per cent of malignant solid tumors in childhood and in patients younger than 10 years is second only to neuroblastoma

The consensus is now that it is malignant tumor of the metanephric renal blastema and that originates in the undifferentiated mesoderm that gives rise to both the nephrons and interstitial elements of the kidney Due to its embryologic and histologic peculiarities, Wilms tumor has at least 50 synonyms The most commonly used terms are nephroblastoma, embryoma, and carcinosarcoma

Approximately two-thirds of the cases occur between the ages of 1 and 5 years, and there is no apparent sex predominance

The first symptoms are either abdominal mass or hematuria In small proportion of cases, the tumors are confined to the kidney and nephrectomy is curative In the majority of cases, they are fatal either because of local extension or metastases

Wilms' tumors may spread to any organ or any of the body cavities The most common metastatic sites are the lung liver mediastinal and retroperitoneal lymph nodes The cure rate largely depends on the clinical stage of the disease, the effective use of pre- and post-operative radiation, and chemotherapy [1,2]

Grossly these tumors are generally large, ovoid, intra-renal masses, which distort and invade the adjacent renal tissue and renal capsule (Fig 1) They are usually unilateral, but bilateral tumors are rarely encountered

The most distinctive histologic features in most tumors are abortive or embryonic glomerulo-tubular structures in an immature spindle cell stroma (Fig 2)

The present study was undertaken to define and to compare the characteristic cytologic features of exfoliated cells of primary and metastatic Wilms tumors, The aim of this review is to call attention to the grave prognostic implication of positive cytologic findings

MATERIALS AND METHODS

During period of 12 years (1958–1969) 56 cytologic specimens from 32 patients were seen in the Cytology Service of Memorial Hospital and interpreted as positive for malignant cells consistent with Wilms tumor Most were pleural or peritoneal effusions, but occasionally tumor cells were identified in urine and sputum specimens

Clinical records and follow-up data were studied, and all available gross and histologic material re-examined

RESULTS

All 32 patients were children under the age of 16 The youngest patient was an eleven month old girl Fourteen were male, and 18 were female (Table I) The right and left kidneys were involved with almost equal frequency All of these patients underwent nephrectomy radiation and chemotherapy in various combinations All but three of the patients died of the disease, on the average within 3 7 months after the initial positive cytologic diagnosis Two of the 3 patients are alive without apparent evidence of disease 13 and 15 months after the first positive cytologic diagnosis was made on ascitic fluid shortly after nephrectomy and both patients subsequently underwent extensive radiation and chemotherapy (Table II) The remaining patient had a positive urine specimen only prior to nephrectomy

In 17 of the 32 cases, the first positive cytologic diagnosis was made on pleural fluid, in nine cases, peritoneal fluid in three cases, sputum, and in three cases, urine (Table III)

The smears of fluids were quite cellular The tumor cells were similar to anaplastic tumor cells seen in tissue sections of this neoplasm (Fig 3a) They were usually present singly rather than in clusters, and they were seen at various stages of differentiation The tumor cells were predominantly oval or short and spindle-shaped cells with

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Exfoliative Cytology of Primary and Metastatic Wilms Tumors

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Wilms' tumor constitutes about 6 per cent of all renal cancers and is the most common renal tumor in childhood It accounts for approximately 30 per cent of malignant solid tumors in childhood and in patients younger than 10 years is second only to neuroblastoma

The consensus is now that it is malignant tumor of the metanephric renal blastema and that originates in the undifferentiated mesoderm that gives rise to both the nephrons and interstitial elements of the kidney Due to its embryologic and histologic peculiarities, Wilms tumor has at least 50 synonyms The most commonly used terms are nephroblastoma, embryoma, and carcinosarcoma

Approximately two-thirds of the cases occur between the ages of 1 and 5 years, and there is no apparent sex predominance

The first symptoms are either abdominal mass or hematuria In small proportion of cases, the tumors are confined to the kidney and nephrectomy is curative In the majority of cases, they are fatal either because of local extension or metastasis

Wilms tumors may spread to any organ or any of the body cavities The most common metastatic sites are the lung, liver mediastinal and retroperitoneal lymph nodes The cure rate largely depends on the clinical stage of the disease, the effective use of pre- and post-operative radiation, and chemotherapy

Grossly these tumors are generally large ovoid, intra-renal masses, *which distort and invade the* adjacent renal tissue and renal capsule (Fig 1) They are usually unilateral, but bilateral tumors are rarely encountered

The most distinctive histologic features in most tumors are abortive or embryonic glomerulo-tubular structures in an immature spindle cell stroma (Fig 2)

The present study was undertaken to define and to compare the characteristic cytologic features of exfoliated cells of primary and metastatic Wilms tumors The aim of this review is to call attention to the grave prognostic implication of positive cytologic findings

Materials and Methods

During period of 12 years (1958–1969) 56 cytologic specimens from 32 patients were seen in the Cytology Service of Memorial Hospital and interpreted as positive for malignant cells consistent with Wilms' tumor Most were pleural or peritoneal effusions, but occasionally tumor cells were identified in urine and sputum specimens

Clinical records and follow-up data were studied, and all available gross and histologic material re-examined

Results

All 32 patients were children under the age of 16 The youngest patient was an eleven month old girl Fourteen were male, and 18 were female (Table I) The right and left kidneys were involved with almost equal frequency All of these patients underwent nephrectomy radiation and chemotherapy in various combinations All but three of the patients died of the disease, on the average within 3 7 months after the initial positive cytologic diagnosis Two of the 3 patients are alive without apparent evidence of disease, 13 and 15 months after the first positive cytologic diagnosis was made on ascitic fluid shortly after nephrectomy and both patients subsequently underwent extensive radiation and chemotherapy (Table II) The remaining patient had positive urine specimen only prior to nephrectomy

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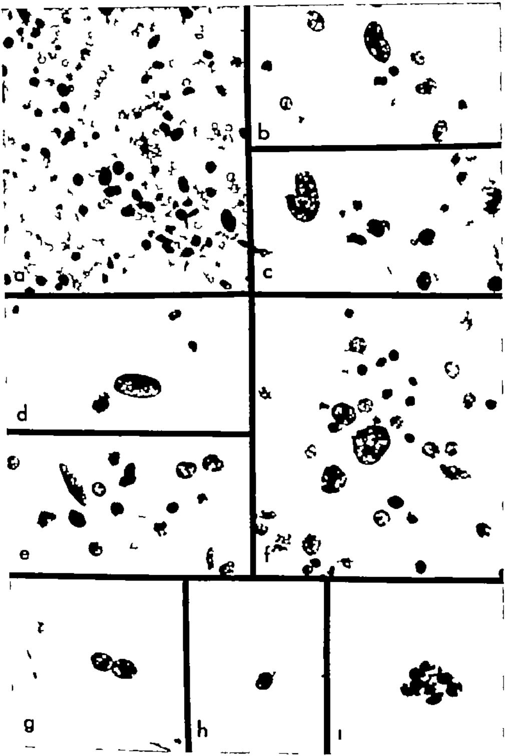

FIG. 3. Exfoliated cells of primary and metastatic Wilms' tumors stained with Papanicolaou stain. Tumor cells of metastatic Wilms' tumors in pleural and peritoneal fluids A, Undifferentiated round cells (× 400). B C, F Oval and spindle cells of various sizes with scant cytoplasm which characterize the tumor cells in effusions (× 560) D E, Cigar-shaped cells with diffusely dispersed, fine nuclear chromatin and prominent nucleoli resembling smooth muscle cells (× 560) G Smear of sputum showing anaplastic tumor cells with fine nuclear chromatin (× 560). H Exfoliated malignant tumor cells of primary Wilms' tumors in urine prior to nephrectomy (× 560). I Small round and oval hyperchromatic nuclei singly or in clusters. Note the minute cytoplasm of the tumor cells and their resemblance to tumor cells in Fig 2 (× 560)

no distinct cytoplasm (Fig 3b c) Round cells with undifferentiated cytoplasm were relatively few The nuclei were usually single, hyperchromatic, and oval or elongated in shape. Chromatin was distributed diffusely throughout the nuclei with occasional condensation at the periphery (Fig 3d, e) The nuclear membrane was usually distinct, sharply demarcated with slightly irregular membrane contours. Nucleoli appeared prominent and they were mostly close to the nuclear membranes. Mononuclear giant cells singly or in clusters were seldom seen (Fig 3f) Cells in mitosis were extremely rare.

Tumor cells in sputum (Fig 3g) and urine (Fig 3h i) were scanty They were, however similar to cancer cells observed in effusions.

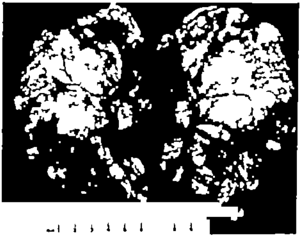

FIG 1 Wilms tumor of the left kidney of 2 year old boy Cut surface of the kidney shows that almost the entire kidney is replaced by the tumor the capsule of the kidney is invaded by the tumor

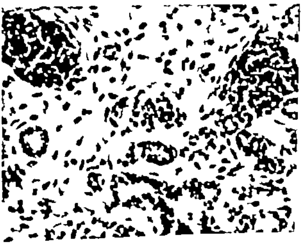

FIG 2 Histologic section of a Wilms tumor show the abortive glomerulo-tubular structures and the anaplastic spindle cells of the stroma Hematoxylin and eosin × 100.

TABLE I Age and Sex Distribution of Patients

Age (years)	Number of patients	
	Male	Female
Under 1	0	1
1 5	12	10
6+	2	7
Total	14	18

TABLE II Prognostic Significance of Positive Cytologic Findings in Metastatic Wilms Tumors

	No. of patients	Average interval (mos.) between	
		Primary tissue diagnosis and 1st positive cytology	1st positive cytology and end of follow-up
Alive	2	1 2	14
Dead	29	1 2	3 7

TABLE III Cytologic Diagnosis of 32 Wilms' Tumors by Type of Specimen

	No. of patients	No. of specimens
Pleural fluid	17	34
Peritoneal fluid	9	15
Urine	3	4
Sputum	3	3
Total	32	56

DISCUSSION

The average interval between time of primary tissue diagnosis and first positive cytology was 12.2 months (Table II) The longest interval, 3 years and 7 months, was observed in a 5 year old boy and the shortest, one day in a 2 year old girl.

Most of the patients had repeated positive cytologic and tissue diagnoses from the metastatic site. On the average, death followed the first positive cytologic report in 3 7 months (Table II)

The resemblance between individual cells of Wilms tumor and normal-developing" renal cells has been demonstrated. However cell groupings that correspond to immature glomerulo-tubular structures can be observed only rarely in smears.

In children, the solitary oval, round and spindly cells of Wilms tumor are rarely imitated by other malignant neoplasms. Neuroblastoma, Ewing's sarcoma, and embryonal rhabdomyosarcoma are the most common cancers which should be considered in the differential diagnosis. Their differ-

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Preparation of Cytologic Smears from Solid Tumors

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Comparison of cytologic preparations with tissue sections has been the foundation of quality control in laboratories of cytopathology In addition, cellular preparations produced by touching or scraping tumor surfaces have been utilized These have however been subject to frequently unacceptable artefacts

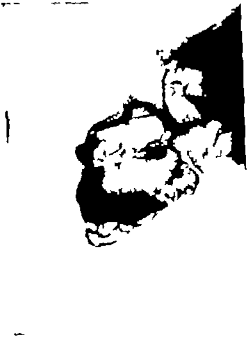

Fig 1 Bronchiolar carcinoma × 1200

Our laboratory has found more satisfactory alternative in technique derived from tissue explant experiments.

Materials and Methods

A small fragment of tumor is washed in saline and then minced with sharp scissors in a petri dish containing 1 1 solution of normal saline and 50% ethyl alcohol. The suspension of washed cells produced is decanted leaving the larger fragments behind. Centrifugation of the suspension t top speed in Model HN International Centrifuge for three minutes yields button which is then spread gently between albuminized slides with frosted surfaces There are then dipped in celloidin for 30 seconds, dried for 30 seconds, and fixed in 95% alcohol for t least 15 minutes prior to staining by the Papanicolaou method.

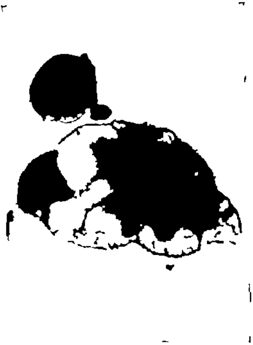

Fig. 2. Bronchiolar carcinoma × 1700

This study was supported in part by U S P H S Grant # 97993

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This paper was presented at the 18th Annual Meeting of the American Society of Cytology Los Angeles, November 1970 entiation from Wilms' tumor may be difficult. However the tumor cells of Ewing's sarcoma show less tendency to form elongated and bizarre spindle cells than those of Wilms' tumor. Tumor cells of neuroblastoma are usually fairly uniform round cells with fine nuclear chromatin and occasional rosette formation.[7] Cells of embryonal rhabdomyosarcoma are small and dark with one or more fine nuclear indentations.[3]

The specific identification of tumor cells may be of importance not only to accurate clinical staging of the disease, but to the initiation of intensive radiation and chemotherapy.[2, 8]

SUMMARY

Cytologic features of primary and metastatic Wilms' tumors seen in smears stained with the Papanicolaou stain, were presented and discussed in a series of 32 cases. The cytology of metastatic Wilms' tumor is usually sufficiently characteristic to make specific cytologic diagnosis possible. The identification of cells of metastatic Wilms' tumor in pleural and peritoneal effusions is usually of grave prognostic significance.

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RESULTS

Figures 1 and 2 are typical of isolated and clustered cells in smears prepared from bronchiolar carcinoma. The cells contain vacuoles and moulded nuclei

Fig. 7. Choriocarcinoma × 480

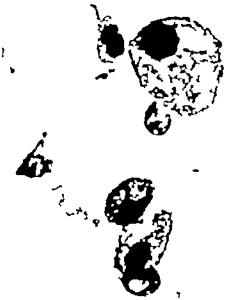

Fig. Smear from tumor. Clear cell carcinoma of kidney × 1200

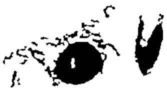

Fig. 8. Sputum. Clear cell carcinoma of kidney × 1200.

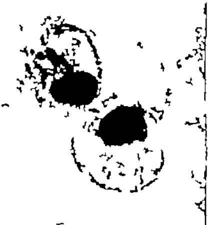

Fig. 10. Ascitic fluid. Liposarcoma. × 1200

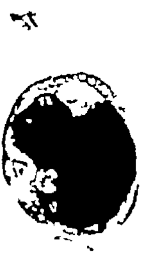

Fig. 3 Adenocarcinoma of colon × 1200

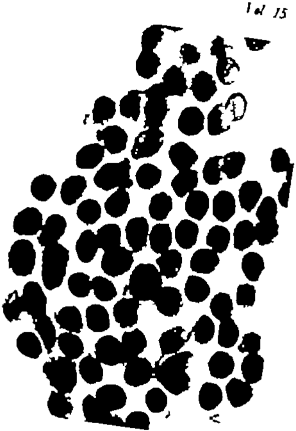

Fig. 5 Fibroadenoma of breast. × 1200

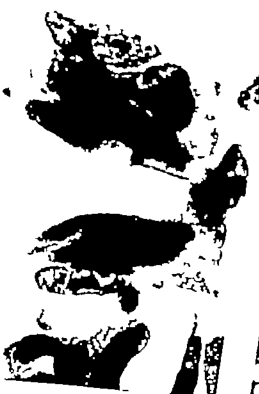

Fig. 4 Adenocarcinoma of colon. × 1200.

Fig. 6 Infiltrating duct carcinoma of breast × 1200

RESULTS

Figures 1 and 2 are typical of isolated and clustered cells in smears prepared from bronchiolar carcinoma. The cells contain vacuoles and moulded nuclei.

FIG. 7. Choriocarcinoma. × 480.

FIG.) Smear from tumor. Clear cell carcinoma of kidney. × 1200.

FIG. 8. Sputum. Clear cell carcinoma of kidney. × 1200.

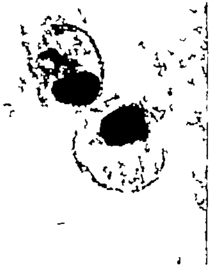

FIG. 10. Ascitic fluid. Liposarcoma. × 1200.

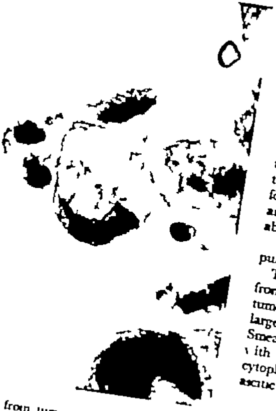

FIG 11 Smear from tumor Liposarcoma × 1200

Cells from an adenocarcinoma of the colon include an isolated cell with a bizarre nucleus and macronucleolus (Fig 3) and closely applied columnar cell in groups (Fig 4)

The cluster of uniform cell with homogeneous spherical nuclei (Fig 5) is derived from a fibroadenoma of the breast

In contrast, smears containing the pleomorphic cells with coarsely granular chromatin in Figure 6 were produced from an infiltrating ductal carcinoma.

The gigantic tumor cell in Figure 7 is representative of a choriocarcinoma of the uterus The nucleus approximates 100 micra in length Detail of its structure are well-preserved despite prior fixation for some time in formalin.

The utility of these preparations in teaching and quality control are illustrated by the following

Figure 8 depicts a cell from the sputum of a patient later found to have a clear cell carcinoma of the kidney At the time, the pattern was considered atypical, but not diagnostic.

Smears from the tumor were prepared at the time of nephrectomy (Fig 9) Cells resembling that in the sputum are present. These elongate forms are not as common as the large cell with ample, basophilic, vacuolated cytoplasm seen above the former

The patient was subsequently proved to have pulmonary metastases.

The cells in Figure 10 were found in ascitic fluid from a terminal patient. The true nature of the tumor was not appreciated until autopsy when a large intra-abdominal liposarcoma was found. Smears from the tumor revealed spherical cells with eccentric nuclei and coarsely vacuolated cytoplasm (Fig 11) corresponding to those in the ascitic fluid.

CONCLUSION

These preparations as illustrated, simulate better than material obtained by touch or scrape routine smears encountered in diagnostic cytology

The value of this method is enhanced by the feasibility of applying it to formalin-fixed tissue.

A comprehensive library of such preparations will prove invaluable for teaching and quality control.

ACKNOWLEDGMENT

The authors wish to thank Dr John M Hinman, J (now deceased) and other members of the resident staff of Harborview Medical Center's Department of Pathology for their enthusiastic support of this project The secretarial assistance of Miss Gladys Lunborn is also gratefully acknowledged

Careers in Cytotechnology—Seventeen Years Experience

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Frequent references have been made to the reduced incidence of invasive cervical carcinoma in Connecticut,[1-] concomitant with increasing application of Cytology as routine screening method. In 1945, only few clinicians and pathologists foresaw the tremendous potential of this new tool for early detection of cervical lesions. As more clinicians saw its value and the female population became more aware of its existence, there has been an increasing demand for trained cytotechnologists Hartford Hospital Cytology Laboratory originally established as service laboratory has gradually assumed increasing responsibility as training facility

It is the purpose of this paper to trace the growth of Hartford Hospital School of Cytotechnology and to assess its contribution to increasing the work force of qualified cytotechnologists Questionnaires were circulated to the 103 graduates of the school with 101 responses From these, data has been tabulated relative to post-graduate contribution to cytotechnology

HISTORICAL BACKGROUND

Hartford Hospital Cytology Laboratory was established in 1945 by the late Ralph Kendall, M D under the auspices of the American Cancer Society In 1952, it became one of 15 laboratories in the United States approved by the American Cancer Society and The Council on Medical Education of the American Medical Association for fellowships in exfoliative cytology In 1958 the program was approved by the American Society of Clinical Pathologists The laboratory has retained approval of these agencies

During this period, methods of recruiting, funding, and training have changed considerably Prior to 1962, newspaper advertisement was the primary means of recruiting trainees to fill anticipated laboratory needs Personnel of other laboratories were sent to us for training Forty-six cytotechnologists and two pathologists trained in this laboratory were funded by the referring institutions Two technologists were subsidized by Connecticut Cancer Society Scholarships From 1945 through 1951 training was relatively informal, chiefly by means of individual instruction by the department supervisor Cytology specimens from patients with benign, abnormal or malignant conditions were collected for teaching material. In conjunction with textbooks, these were used as the basic content of the curriculum

In 1952, more formally structured curriculum was developed under the guidance of the late Elizabeth S Wheeler Ph D A course syllabus was printed to guide students through all phases of diagnostic cytology at pace suitable to the individual student Prior to 1955 individual students were admitted at irregular intervals and graduated when judged to be qualified. Often two or three students would enter simultaneously Lectures were added as more interest in the method developed among clinicians and pathologists and more teaching material became available Members of the pathology department were scheduled to participate more formally and actively in the teaching, giving background lectures and cytopathological correlations as each organ system was studied In 1957 three senior cytotechnologists assumed some of the teaching responsibilities, primarily in using specialized microprojector an invaluable aid for group instruction to assure that all students see the same specimen details simultaneously

In 1962, the need for cytotechnologists throughout the country had become so great that the United States Public Health Service provided funds to subsidize training programs Hartford Hospital was selected to train maximum of eight students per year under Cytotechnology Training Grant #TO-17008 This was increased to 16 per year in 1965 with expansion of the physical facilities to accommodate class of eight twice year The original grant from the USPHS supplied funds to cover a stipend of $225 per month per student for six months of didactic training, a portion of the salaries and the entire cost of continuing education for the teaching personnel, as

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well as projected expenses for the modern educational material needed to cope with the demand for more sophisticated training. As changes in the format for schools operating under USPHS funds developed, the terms and conditions stated that a *training allowance not to exceed $1,800* be granted the school for each student up to the enrollment capacity for one class. For each additional student an amount not to exceed $1,100 per student was *granted the institution. No funds* were available for the six month apprenticeship period. Graduates who could not be accommodated under the Hartford Hospital Cytology Laboratory man-hour budget were referred to institutions willing to accept them as apprentices at the minimum rate for cytotechnologists.

In 1969, in keeping with the national trend, the program was expanded to 12 months with the USPHS underwriting eight student stipends for a full year rather than 16 for six months. This full year's program, maintaining the schedule of six months didactic training and six months apprenticeship, assured a continuing educational experience in a work situation that provided a high volume of varied material. The trainee was carefully supervised, all work was checked by a senior cytotechnologist and any discrepancies were discussed. This prevented the possibility of an apprentice assuming a responsibility for which she was not fully prepared. Unfortunately such supervision was not appreciated by a few who felt qualified to assume a service function at service pay as had been the previous practice. However, the majority felt that during this period they learned the finer points of diagnostic cytology.

Because the federal support would be available to us in 1963, a more active recruitment policy was adopted early in 1962. Applicants were sought through the placement offices of junior and senior *colleges that had previously* supplied well-qualified general medical technologists to Hartford Hospital. In 1966, Quinnipiac College, a Connecticut coeducational college, instituted a two year Associate in Science degree program to provide the specific college background required by the ASCP for students planning to enter approved Schools of Cytotechnology. In 1967, a feature article in a Hartford newspaper brought over 900 inquiries with a dozen applications.

Admission to the school has been dependent on several factors:

1. Submission of a formal application
2. Submission of an official college transcript
3. Evaluation of the transcript by the ASCP
4. Satisfactory personal interview
5. Letters of recommendation
6. Agreement to complete one year of training and one year of service
7. Agreement to take the National Registry Examination.

One hundred fifty-seven applications have been received since 1962 and 61 students have actually entered our program with 55 completing their training under the Public Health Training Grant.

RESULTS

Since 1952, 98 women and five men have satisfactorily completed our six month program of didactic training. A questionnaire sent to these 103 graduates provided the basis for determining how far reaching our program has been. Satisfactory data is available on 101 of these graduates.

Residents of nine states and two foreign countries graduated from our program. They represent colleges in 19 states and three foreign countries (Table I). Thirty four have baccalaureate degrees, 32 associate degrees, 34 have college credits without degrees. *Three trained prior to 1960 have no college background.*

The majority of our students came to us directly from college, but many entered the program after several years in other professions or homemaking.

Our currently employed graduates are located in 11 states and Puerto Rico. One is deceased and only one is completely untraceable. Forty-seven are employed full-time and 11 part-time (Table II). Four who work full-time also work part-time for smaller laboratories. One does part time screening for two laboratories.

TABLE I *Colleges Attended*

Location	Number
Connecticut	39
Massachusetts	12
Vermont	6
New Hampshire	5
Maine	4
Other states	19
More than 1	8
Foreign	4
None	3
Unknown	3
Total	103

TABLE II *Current Employment*

State	No.	Full-time	Part-time
Connecticut	39	31	11
Massachusetts	6	6	1
Colorado	2		0
Rhode Island	3	2	1
California	1	1	1
Puerto Rico	1	1	0
Other states (6)	6	4	
Total	58	47	16

The current positions held by this group are shown in Table III. The total of 72 is due to the fact that the majority of supervisors, teachers, and assistant teachers also function as screeners. Forty-nine hospitals and 14 private laboratories are represented.

In a field with a predominance of women, concern is often expressed as to how long graduates will remain active. As demonstrated in Table IV most have remained long enough to make a substantial contribution. It is particularly notable that the figures in Table V indicate older students serve more productive years after graduation than younger ones. The major reason for abandoning active work is pregnancy (Table VI). The five who entered other professions have remained in paramedical fields, one in hematology, two in cytogenetics, and two in cardio-pulmonary laboratories.

Fifty-five graduates remained with Hartford Hospital for their six month apprenticeship. Forty-eight served their apprenticeship in other laboratories approved for training by the director of Hartford Hospital Laboratory. Approval is dependent on supervision by a registered cytotechnologist and a pathologist with experience and genuine interest in cytology. In two instances, hospitals within commuting distance of Hartford had dire need of screeners but could not meet the above qualifications. The apprentice was then supervised by senior personnel at Hartford Hospital although the screened specimens from the laboratory employing her.

TABLE III *Current Positions*

Screeners	51
Supervisors	10
Teachers	2
Assistant teachers	3
Students apprentices	6
Total	72

TABLE IV *Time Employed in Cytology*

Less than 6 months	6
6 months–2 years	33
5 years	25
5–10 years	18
Over 10 years	11
Unknown	10
Total	103

TABLE V *Work Longevity vs Age at Time of Training*

Age	Number	Years
19–25	81	4.5
25–30	7	5.0
30–35	5	6.4
35–40	5	7.0
40–55	5	8.1

TABLE VI *Reasons for Leaving Field*

Family	22
Different field	5
Further education	2
No available job	4
Dislike	1
Health	1
Retired	1
Death	1
Unknown	2
Total	39

Fifty-five graduates are ASCP registered cytotechnologists five have taken the examination but we do not have official notification as to their status. Seven recently completed their requirements and will take the examination next March. The majority of those who did not take the ASCP certifying examination feel that they have job security without certification.

Few of our graduates have any professional affiliation. Only 12 belong to the American Society of Cytology. One belongs to the International Society of Cytology and two are members of organized state cytology groups. Although Connecticut cytotechnologists and pathologists hold two well-attended meetings per year there is no formal organization.

In 1969 Public Health Service support was withdrawn. The Department of Allied Health at Hartford Hospital allocated funds to provide a student stipend of $125 per month per student plus a portion of other operating costs. With increasing demands on the hospital budget, there is a distinct possibility that we may have to revert to training students only for our own needs. It is anticipated that this will have a long-term effect on the supply of trained cytotechnologists in the New England area. Ultimately it may produce a quality effect by inability to keep pace with educational training aids and continuing education needs of the teaching supervisors.

SUMMARY

Over the past 17 years, Hartford Hospital has graduated 103 cytotechnologists. Together they have contributed 477.5 years of active service in the field of diagnostic cytotechnology. Fifty-four of our graduates have worked at least two years and twenty have contributed six or more productive years. Although the number of women trained after the age of 31 is low (15) they are the ones who have made the most substantial contribution in duration of service.

well as projected expenses for the modern educational material needed to cope with the demand for more sophisticated training As changes in the format for schools operating under USPHS funds developed, the terms and conditions stated that a training allowance not to exceed $1 800 be granted the school for each student up to the enrollment capacity for one class For each additional student an amount not to exceed $1 100 per student was granted the Institution. No funds were available for the six month apprenticeship period. Graduates who could not be accommodated under the Hartford Hospital Cytology Laboratory man-hour budget were referred to Institutions willing to accept them as apprentices at the minimum rate for cytotechnologists.

In 1969 in keeping with the national trend the program was expanded to 12 months with the USPHS underwriting eight student stipends for a full year rather than 16 for six months. This full year's program, maintaining the schedule of six months didactic training and six months apprenticeship assured a continuing educational experience in a work situation that provided a high volume of varied material. The trainee was carefully supervised, all work was checked by a senior cytotechnologist and any discrepancies were discussed. This prevented the possibility of an apprentice assuming a responsibility for which she was not fully prepared Unfortunately such supervision was not appreciated by a few who felt qualified to assume a service function at service pay as had been the previous practice However the majority felt that during this period they learned the finer points of diagnostic cytology

Because the federal support would be available to us in 1963 a more active recruitment policy was adopted early in 1962 Applicants were sought through the placement offices of junior and senior colleges that had previously supplied well-qualified general medical technologists to Hartford Hospital. In 1966, Quinnipiac College, a Connecticut coeducational college, instituted a two year Associate in Science degree program to provide the specific college background required by the ASCP for students planning to enter approved Schools of Cytotechnology In 1967 a feature article in a Hartford newspaper brought over 900 inquiries with a dozen applications

Admission to the school has been dependent on several factors

1 Submission of a formal application
2. Submission of an official college transcript
3 Evaluation of the transcript by the ASCP
4 Satisfactory personal interview
5. Letters of recommendation
6. Agreement to complete one year of training and one year of service
7 Agreement to take the National Registry Examination.

One hundred fifty-seven applications have been received since 1962 and 61 students have actually entered our program with 55 completing their training under the Public Health Training Grant.

RESULTS

Since 1952, 98 women and five men have satisfactorily completed our six month program of didactic training A questionnaire sent to these 103 graduates provided the basis for determining how far-reaching our program has been. Satisfactory data is available on 101 of these graduates.

Residents of nine states and two foreign countries graduated from our program. They represent collages in 19 states and three foreign countries (Table I) Thirty-four have baccalaureate degrees, 32 associate degrees, 34 have college credits without degrees. Three, trained prior to 1960 have no college background.

The majority of our students came to us directly from college but many entered the program after several years in other professions or homemaking

Our currently employed graduates are located in 11 states and Puerto Rico. One is deceased and only one is completely untraceable. Forty seven are employed full-time and 11 part time (Table II) Four who work full-time also work part time for smaller laboratories. One does part-time screening for two laboratories.

TABLE I *Colleges Attended*

Location	Number
Connecticut	39
Massachusetts	12
Vermont	6
New Hampshire	5
Maine	4
Other states	19
More than 1	8
Foreign	4
None	5
Unknown	3
Total	103

TABLE II *Current Employment*

State	N	Full time	Part time
Connecticut	39	31	11
Massachusetts	6	6	1
Colorado		2	0
Rhode Island	3		1
California	1	1	1
Puerto Rico	1	1	0
Other states (6)	6	4	2
Total	58	47	16

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A State Wide Proficiency Testing Program in Cytology

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Laboratory evaluation programs have as their primary goal to upgrade the quality of performance of all participants Evaluation programs attempt to monitor and at the same time improve, the accuracy with which specimens are identified or analyzed. If laboratory results are to be reliable all phases of the laboratory operation must be carried out in carefully-controlled system, commonly referred to as the internal quality control program. Laboratory evaluation, in a sense, serves as an external quality control, monitoring the multiple elements of this system, including personnel proficiency supervision and direction, facilities and equipment maintenance, records processing, specimen handling and procedures performance While internal quality control programs can uncover problems in the day-to-day operation of laboratory evaluation programs provide the director with an independent, unbiased estimation of his laboratory total operation In addition, state and federal programs have often established minimal standards for the various phases of laboratory practice, and they carry out laboratory inspection at regular intervals to determine whether participants are complying with standards and with the general rules and regulations

Another phase of laboratory evaluation is concerned with assessing the accuracy with which diagnostic tests are performed One approach would be to determine the validity of laboratory test results by system of spot-checking and sampling, with analyses run in duplicate by the evaluation personnel Such system, carried out on regular basis, would be extremely time-consuming and would have several major practical limitations The method employed by most programs is based on series of unknown samples mailed to participating laboratories for analysis, system known as proficiency testing Individual laboratory analyses are compared with results obtained on the same specimen by reference laboratories of recognized competence and/or by referee peer laboratories who have demonstrated high degree of proficiency

A major objective of proficiency testing is to provide quantitative grading system for regulating clinical laboratories A broader purpose as defined in November 1967 by the American Association of Clinical Chemists, states that the ultimate objective should be attainment of true results for the constituent being determined. This position paper further states that proficiency testing results should

1 Compare participants results with true results insofar as the current state-of-the art permits
2. Compare results with those of other laboratories for test specimens from the identical lot, and
3 Consider the degree of accuracy ordinarily required for meaningful clinical application.

There are several practical and theoretical drawbacks to the usual practice of proficiency testing The most serious objection is that test specimens are identified as such, and they are often treated as "special" samples rather than routine ones Thus, the most competent technologist may be assigned to perform the analysis, and the determination may be carried out several times Ideally proficiency specimen should be introduced into the daily workload and handled as an ordinary patient sample To achieve this objective requires total understanding, cooperation, and commitment from the participants. A technique used by several programs to preclude special handling is to carry specimens to the laboratory and to observe the personnel actually perform the analyses This system is still not ideal, since it may not measure ordinary laboratory performance A preferable system is the "blind" submission of samples which are disguised as patient specimens Only the logistics of the latter approach prevent it from being utilized more extensively

Proficiency testing, as carried out by most programs, generally measures laboratory's best performance The technique by itself cannot be expected to detect all the deficiencies of laboratory let alone provide complete profile of the laboratory operation Proficiency testing, nevertheless, is mechanism for determining whether laboratories have major deficiencies in specific areas. To be most effective proficiency testing should be incorporated with inspection into comprehensive laboratory improvement program

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The importance of adequate external funding of Schools of Cytotechnology cannot be overlooked if we are to meet the continuing demand for well trained cytotechnologists.

ACKNOWLEDGMENT

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vaginals), presence of blood, inflammatory cells, micro-organisms, and other observations used to make a diagnosis. The person performing the examination, the supervisor and the director of the laboratory are asked to sign the report—these may be one, two, or three individuals, depending on the individual laboratory.

Slides are identified as follows: C-11 1 5

C = Cytology
11 = Survey number
1 = Slide number in the survey
5 = Identification number of the slide

The identification number permits retrieval of specific slide when questions are asked by an individual laboratory. This is important since some specimens, including all cervical-vaginals, are selected from individual comparable cases, matched as closely cytologically and clinically as possible. As an example, in one survey case, slides were selected which displayed atypia and heavy trichomonas infection in young women. Other specimens may be from one patient, such as body fluids, urines, sputums, and gastrics, where it is possible to make multiple slides or filters from single or repeat specimens. The first method of test sample preparation presents a problem unique to cytology since it is not possible to submit aliquots from a single pool. Matched samples must be carefully selected to demonstrate certain features, yet they necessarily reflect the practices and standards of the control laboratory, in this case the State Laboratory of Hygiene. Because of the difficulties associated with matching cases, the program has been limited to the fifteen independent clinical laboratories in Wisconsin, although many hospital laboratories have expressed an interest in participating. To circumvent the problems of matching cases, evaluation surveys could be carried out on a different basis. A large central repository of coded slides could be developed as a source from which slides are mailed or hand-carried to laboratories on a regular or intermittent basis. This system would not be comparable to the usual proficiency survey but it could provide useful information on laboratory performance. Projection slides or photographs of representative cells could be submitted to laboratories, but in the opinion of the authors, these do not provide a reasonable basis for evaluating laboratory performance.

A principle stated at the outset was that proficiency testing should strive to attain true results for the constituent. Cases are selected after a diagnosis has been established with reasonable certainty. In many instances, the results of tissue diagnosis are available. In other cases, diagnosis may be reached after considering other laboratory or x-ray findings, or on clinical evaluation alone. The evaluation procedure, however, is based on the cytologic diagnostic consensus of the four reference laboratories. (The reference laboratories are Dr Teruo Masukawa, Milwaukee County Hospital; Dr Thomas R. Simon, Syracuse Memorial Hospital; Dr Elizabeth McGrew, University of Illinois, and Dr Stanley L. Inhorn, State Laboratory of Hygiene.) When the consultants do not reach an agreement, the results of that particular survey case are not included in the overall evaluation. The basis for evaluation must be the specimen itself, the cytologic preparation, not necessarily the confirmed tissue diagnosis, since the slide may not always reflect the proven disease entity. As an example, one case showed only mild atypia, yet the women was found on subsequent biopsy to have invasive squamous cell carcinoma.

Performance is judged on the laboratory's estimation of the degree of cellular alteration. The major difficulty is equating the systems used by different laboratories. Generally, these systems are divided into four or five levels, with negative being equivalent to grades 1 or 2, atypical—grades 2 or 3, suspicious—grades 3 or 4, and positive—grades 4 or 5. Laboratories were instructed to report in their usual fashion, being as specific as they wished, but at the same time translating their reports into a four-level system. The results are then graded according to their comparison with the reference laboratories. Where three reference laboratories agree and one is discrepant, that result may be discarded in selected instances.

C.A. or Complete Agreement are those reports that fall within the range reported by the reference laboratories.

P.A. or Partial Agreement are reports where the difference between the participating laboratory and reference laboratories is of a minor degree.

D.A. or Disagreement—a major discrepancy of two or more categories. Thus if the reference laboratories all call a smear positive and a participating laboratory labels it "atypical" this will be a major discrepancy or disagreement.

The method for calculating the overall achievement is given by the following formula:

Percent agreement

$$= \frac{\text{Number C.A.} + \frac{1}{2}\,\text{Number P.A.}}{\text{Total Reports Compared}} \times 100$$

This method has been borrowed from the syphilis serology evaluation procedure. The level of adequate performance for agreement has been established as 90%. Annual summaries for the 16 cases are made for all participating laboratories, and those laboratories not achieving the 90% level are offered assistance and consultation. Provisional approval for the following year may be given in the latter instance.

Summary reports are sent to all laboratories approximately one month after the survey. All laboratories are identified by their code numbers to maintain anonymity. Laboratories can then com-which offers consultation training and technical assistance. With such a program, weaknesses detected during inspection or proficiency testing can be corrected or controlled through the feedback process. A comprehensive program can also determine areas of weakness in laboratory technology regarding reagents procedures, and instrumentation Cumulative experience from evaluation programs can lead to consensus on methodology and can demonstrate to the laboratory director the advantages of one technique over another

For evaluating laboratory test performance several criteria may be utilized

Accuracy refers to the closeness of the participant's result to the correct answer

Precision is the ability of the laboratory to obtain results close to each other on the same sample Precision or reproducibility is usually expressed in terms of standard deviation or coefficient of variation

Trends measure the level of performance of a laboratory over a period of time.

Tendency determines whether a laboratory tends to make certain kinds of errors. The quality of laboratory performance should be defined in measurable terms, and this requires that each laboratory maintain a practical system of continuous quality measurement. Proficiency testing is an external surveillance system which has severe limitations regarding its scope and responsiveness it should never be regarded as a substitute for internal quality control

While the need for evaluating the reliability of clinical laboratory testing has been recognized for many years, few evaluation programs were developed by federal, state, local governments, or by professional societies until the mid-1960's. Passage of the Social Security Act Health Insurance for the Aged (Medicare) in 1966 and the Clinical Laboratories Improvement Act (Interstate Licensure) in 1967 stimulated creation of new programs at all levels. Under the Medicare program, the federal government established minimum standards which independent clinical laboratories had to meet to receive reimbursement for services. Medicare regulations involved all phases of laboratory operation and adopted specific conditions related to the laboratory director supervisor technical personnel records, equipment, facilities, and tests performed In addition to personnel standards, Medicare also emphasized quality control and preventive maintenance. One effect of Medicare was to accelerate the expansion of proficiency testing programs administered by states and professional societies, since Medicare required proficiency testing of non-doctoral operated laboratories.

Materials and Methods

In Wisconsin, the Division of Health has the statutory responsibility to carry out approval of clinical laboratories for disease control Special statutes are concerned with pre-marital serologies, enteric disease control, PKU testing and blood alcohol determinations. The Division of Health can also certify laboratories for other specific examinations Under the Wisconsin Hospital Rules and Regulations, hospital laboratories are inspected, evaluated, and since June 1 1968, they are required to participate in laboratory evaluation programs offered by the Division of Health in those specialties for which the laboratory offers services. In addition to these laws and statutes, the Division of Health has basic public health concern for the quality quantity and availability of laboratory services essential for disease prevention, diagnosis management, and control. It also assists laboratories to apply new knowledge and services and to meet the needs and requirements of federal and state health programs.

When Medicare legislation was enacted, Wisconsin greatly expanded its laboratory evaluation program to permit certification of independent laboratories whose directors did not hold doctorate degrees. Separate programs were developed for each laboratory specialty as defined by the Social Security Administration. Although a program was not required by Medicare for exfoliative cytology since the director or supervisor must be a physician, the Division of Health decided to establish a program for the following reasons. First, a major component of the laboratory practice in cytology is carried out by personnel who are at the technologist level, a situation comparable to laboratory practice in other specialties. Therefore, the requirement of a doctoral director will not in itself guarantee a high level of cytodiagnostic practice. Furthermore, a proficiency testing program in cytology would afford an opportunity to determine weaknesses in the practice of cytology and to determine where corrective action and training would be beneficial.

The Cytology Evaluation Program was started in August, 1967 for the independent clinical laboratories of Wisconsin. Test slides were selected from the specimen files of the Cytology Unit, Pathology Section, of the Wisconsin State Laboratory of Hygiene For the first two years of operation, three cases were submitted on a bi-monthly basis subsequently four cases have been sent quarterly To date, sixteen surveys have been completed Participating laboratories are instructed to evaluate the slides and to report the results on enclosed forms within five days. Slides may be photographed and retained until the summary report is received, which is approximately one month after the survey Slides must be returned since they are actual patient material and not fabricated specimens, as are most survey samples. Reports should include the cytologic grading comments on background compatibility with age (for cervical-vaginals) presence of blood, inflammatory cells, micro-organisms, and other observations used to make a diagnosis. The person performing the examination, the supervisor and the director of the laboratory are asked to sign the report—these may be one, two, or three individuals, depending on the individual laboratory.

Slides are identified as follows: C-11.1.5

C = Cytology
11 = Survey number
1 = Slide number in the survey
5 = Identification number of the slide

The identification number permits retrieval of a specific slide when questions are asked by an individual laboratory. This is important since some specimens, including all cervical-vaginals, are selected from individual comparable cases, matched as closely cytologically and clinically as possible. As an example, in one survey case slides were selected which displayed atypia and heavy trichomonas infection in young women. Other specimens may be from one patient, such as body fluids, urines, sputums, and gastrics, where it is possible to make multiple slides or filters from single or repeat specimens. The first method of test sample preparation presents a problem unique to cytology since it is not possible to submit aliquots from a single pool. Matched samples must be carefully selected to demonstrate certain features, yet they necessarily reflect the practices and standards of the control laboratory, in this case the State Laboratory of Hygiene. Because of the difficulties associated with matching cases, the program has been limited to the fifteen independent clinical laboratories in Wisconsin, although many hospital laboratories have expressed an interest in participating. To circumvent the problem of matching cases, evaluation surveys could be carried out on a different basis. A large central repository of coded slides could be developed as a source from which slides are mailed or hand carried to laboratories on a regular or intermittent basis. This system would not be comparable to the usual proficiency survey, but it could provide useful information on laboratory performance. Projection slides or photographs of representative cells could be submitted to laboratories, but in the opinion of the authors, these do not provide a reasonable basis for evaluating laboratory performance.

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Performance is judged on the laboratory's estimation of the degree of cellular alteration. The major difficulty is equating the systems used by different laboratories. Generally, these systems are divided into four or five levels, with negative being equivalent to grades 1 or 2, atypical—grades 2 or 3, suspicious—grades 3 or 4, and positive—grades 4 or 5. Laboratories were instructed to report in their usual fashion, being as specific as they wished, but at the same time translating their reports into a four-level system. The results are then graded according to their comparison with the reference laboratories. Where three reference laboratories agree and one is discrepant, that result may be discarded in selected instances.

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The method for calculating the overall achievement is given by the following formula:

Percent agreement

$$= \frac{\text{Number C.A.} + \tfrac{1}{2}\ \text{Number P.A.}}{\text{Total Reports Compared}} \times 100$$

This method has been borrowed from the syphilis serology evaluation procedure. The level of adequate performance for agreement has been established as 90%. Annual summaries for the 16 cases are made for all participating laboratories, and those laboratories not achieving the 90% level are offered assistance and consultation. Provisional approval for the following year may be given in the latter instance.

Summary reports are sent to all laboratories approximately one month after the survey. All laboratories are identified by their code numbers to maintain anonymity. Laboratories can then compare their results with their peer laboratories and with the consultants In addition, a report of each case is prepared by the program director which summarizes the pertinent findings and correlates the cytology with the clinical situation. For each case representative comments from the participants and consultants are edited. *Survey reports* are essential to the success of the program Not only do they permit each laboratory to compare its performance with peer laboratories and those of national reputation, but they provide for positive and negative feedback These reports also supply an important ingredient of this program, namely continuing education, which is an essential to the objective of laboratory improvement

The next consideration is the selection of cases to include in a survey While it is tempting to include slides from various specific types of cancer the obviously positive smears and the very negative smears do not effectively differentiate laboratory performance. Thus one must rely on cases which are equivocal or which display unusual features to elicit variation in reporting There should be a mixture of negative ambiguous, and positive cases to provide good differentiation of participating laboratories. Table I is a summary of the sample material used in the Cytology Evaluation Program 53 test specimens were submitted in the first 16 surveys 24 were in the negative category 8 were atypical, 1 suspicious and 20 positive Slightly more than half were from the female genital tract Regarding the negative cervical vaginals, 13 of the 14 were selected to demonstrate or be associated with trichomonas infection, yeast post menopausal atrophy pregnancy post-partum condition, endocervical cells, giant cells, women on the pill, or leptothrix. *Similarly* the negative sputums were selected from patients with pulmonary conditions such as tuberculosis, pneumothorax, and pulmonary congestion Abnormal cervical-vaginal smears included 1 case of dysplasia, 3 of carcinoma in-situ, 3 invasive squamous cell carcinomas, and 3 adenocarcinomas of the endometrium. A lengthy discussion of the many interesting cases in these surveys is beyond the scope of this paper howeve the educational content should be apparent fro these few examples.

TABLE I *Distribution of Test Specimens*

Specimen source	Negative	Atypical	Suspicious	Positive	Total
Cervical Vaginal	14	7		7	28
Sputum Bronchoscopy	5	1	1	5	1
Body Fluid	3			6	9
Urine	1				3
Spermatocele	1				1
Total	24	8	1	20	53

TABLE II *Evaluation of Cytology Proficiency Testing Surveys C 1 through C 16*

Code Number	Total Reports	Complete Agree.	Partial Agree	Disagree	(Co. Ct. ½ Pa. P) / (Total Reports) X 100
A	48	45	2	1	96
B	43	39	4		91
C	51	40	3	8	81
D	38	33	5		93
E	51	43	8		92
F	36	32	4		94
G	51	45	5	1	93
H	51	33	7	9	75
I	51	50	1		99
J	48	37	7	4	83
K	51	46	4	1	94
L	46	42	1	3	93
M	7	6	1		93
N	1	14	4	3	76
O	8	7		1	87

RESULTS

The overall performance of participating laboratories for the 16 surveys is given in Table II From this table one may note that 10 of the 1 laboratories performed at acceptable levels of performance Laboratory 0 has recently joined th program, so there is insufficient data for adequat evaluation *Laboratory N makes errors in eithe* direction overdiagnosing and underdiagnosing a does laboratory J Laboratory H on the othe hand, tends to overdiagnose frequently which i also true of laboratory C These latter two, therefore tend to make systematic errors, which ha been true throughout the program for laborator H but which is a recent problem for laboratory C perhaps reflecting a change of personnel It shoul be noted that marked discrepancies among th reference laboratories were recorded nine times, which required that the test results be discarded in two cases Of the other seven two outliers were discarded, and in the remaining five results were acceptable in the wider range which resulted

To determine reproducibility in laboratory performance slides have been resubmitted to individual laboratories. The entire Survey C-15, was devoted to a study of reproducibility as is shown in Table III It can be seen that most laboratories tend to be precise or reproducible in their diagnoses, *with certain laboratories* tending to mak consistent errors in evaluation.

TABLE III *Results from Survey C 15*

CODE NUMBER	VAGINAL		PLEURAL		SPUTUM		SPUTUM	
	C-15 1	C-8-1	C-15-2	C-8-2	C 15-3	C-4-	C 15-4	C 9 2
L	NI	AII	SIII	P	NI	NI	SIII	AII
H	S	S	P	N	S	S	P	N
F	N	N	N	N	N		N	
I	A	N	N	A	N	N	N	N
E	A	NII	A	N	N	AII	N	N
A	AIII	NII	NII	NII	NII	NII	SIV	N
G	AII	NII	NI	SIII	NI	II	NI	NI
B	AII	AII	—	N	—	AII	II III	AII
J	NI	N	NI	AII	NI	NII	AII	
K	N	N	N	N	N	A	N	N
C	S	S	P	P	S	P	S	N
N	N		A		S		N	
O	N							
Cons. 1	N	NII	N	A	N	N	N	N
Cons. 2	N	N	N	N	N	A	S	A
Cons 3	A		P*		N		N	
State Lab	N	N	N	N	N	N	N	N

N-negative A-atypical S-suspicious P-positive
Outlier

DISCUSSION

This proficiency testing program in cytology has afforded us the opportunity to determine what role laboratory evaluation may play in diagnostic cytology practice It has also pointed out several problems, many of which remain unresolved Evaluation is designed to test the operation and output of the entire laboratory In cytology however the testing procedure is extremely subjective, depending heavily on the training and experience of the individual cytotechnologist In laboratory where several cytotechnologists are employed, it is impossible for this type of intermittent survey technique to completely evaluate the overall performance of that laboratory especially the intricate interrelationships between screeners, supervisory cytotechnologists, and pathologists. Proficiency testing programs will continue to be plagued by problems in terminology and classification. It can, however afford each laboratory director an opportunity to compare his laboratory operation with that of other cytology units and to determine whether certain questionable practices may benefit from review and corrective action, carried out internally or with assistance from an experienced consultant

It should be emphasized that cytology like cytohematology and certain other laboratory fields, relies heavily on the competence and dedication of the primary benchworker namely the cytotechnologist The basic analytic procedure is highly subjective and good results depend on the education, experience motivation, supervision, and management of the bench personnel. It is obvious then that cytology laboratory employing several cytotechnologists is only as good as its poorest technologist Since external laboratory evaluation can only measure final laboratory output, continuous high quality performance must be assured by comprehensive program of internal quality control which emphasizes personnel monitoring Whereas some fields, like clinical chemistry and immunology are dependent on careful equipment maintenance, the employment of standards and control sera, and use of certified reagents, cytology must emphasize excellence in microscopic specimen evaluation.

Insistence on high personnel standards and professionalism in cytotechnology must be the key stones to quality in diagnostic cytology Improvement in the curriculum of schools of cytotechnology has added immeasurably to upgrading personnel quality It would appear however that there has been little attention paid to the quality control aspects of cytotechnology The Medicare regulations recognize the importance of the laboratory director in the practice of exfoliative cytology In addition to recognizing the board-certified pathologist, Medicare designated the American Society of Cytology to develop an examination in cytopathology for those not otherwise qualified. Equivalent qualifications for certification may also be recognized by the Social Security Administration The Clinical Laboratories Improvement Act of 1967 (Interstate Licensure) places greater emphasis on quality control, although in exfoliative pare their results with their peer laboratories and with the consultants. In addition, a report of each case is prepared by the program director which summarizes the pertinent findings and correlates the cytology with the clinical situation. For each case representative comments from the participants and consultants are edited. Survey reports are essential to the success of the program. Not only do they permit each laboratory to compare its performance with peer laboratories and those of national reputation but they provide for positive and negative feedback. These reports also supply an important ingredient of this program, namely continuing education, which is an essential to the objective of laboratory improvement.

The next consideration is the selection of cases to include in a survey. While it is tempting to include slides from various specific types of cancer the obviously positive smears and the very negative smears do not effectively differentiate laboratory performance. Thus one must rely on cases which are equivocal or which display unusual features to elicit variation in reporting. There should be a mixture of negative, ambiguous, and positive cases to provide good differentiation of participating laboratories. Table I is a summary of the sample material used in the Cytology Evaluation Program. 53 test specimens were submitted in the first 16 surveys. 24 were in the negative category, 8 were atypical, 1 suspicious, and 20 positive. Slightly more than half were from the female genital tract. Regarding the negative cervical-vaginals, 13 of the 14 were selected to demonstrate or be associated with trichomonas infection, yeast, post-menopausal atrophy, pregnancy, post-partum condition, endocervical cells, giant cells, women on the pill, or leptothrix. Similarly the negative sputums were selected from patients with pulmonary conditions such as tuberculosis, pneumothorax, and pulmonary congestion. Abnormal cervical vaginal smears included 1 case of dysplasia, 3 of carcinoma-in-situ, 3 invasive squamous cell carcinomas, and 3 adenocarcinomas of the endometrium. A lengthy discussion of the many interesting cases in these surveys is beyond the scope of this paper, however the educational content should be apparent from these few examples.

TABLE I. *Distribution of Test Specimens*

Specimen source	Negative	Atypical	Suspicious	Positive	Total
Cervical Vaginal	14	7		7	28
Sputum	5	1	1	5	[illegible]
Bronchoscopy					
Body Fluid	3			6	[illegible]
Urine	1				3
Spermatocele	1				1
Total	4	8	1	20	53

TABLE II. *Evaluation of Cytology Proficiency Testing Surveys C-1 through C-16*

Code Number	Total Reports	Complete Agree.	Partial Agree.	Disagree	(No. CA + ½ No. PA) / (Total Reports) × 100
A	48	45	2	1	96
B	45	39	4		91
C	51	40	3	8	81
D	38	33	5		93
E	51	43	8		92
F	36	32	4		94
G	51	45	5	1	93
H	51	35	7	9	75
I	51	50	1		99
J	48	37	7	4	83
K	51	46	4	1	94
L	41	4[illegible]	1	3	93
M	7	6	1		93
N	1	14	4	3	76
O	8	7		1	87

RESULTS

The overall performance of participating laboratories for the 16 surveys is given in Table II. From this table one may note that 10 of the 15 laboratories performed at acceptable levels of performance. Laboratory O has recently joined the program, so there is insufficient data for adequate evaluation. Laboratory N makes errors in either direction, overdiagnosing and underdiagnosing as does laboratory J. Laboratory H, on the other hand, tends to overdiagnose frequently which is also true of laboratory C. These latter two, therefore, tend to make systematic errors, which has been true throughout the program for laboratory H but which is a recent problem for laboratory C, perhaps reflecting a change of personnel. It should be noted that marked discrepancies among the reference laboratories were recorded nine times, which required that the test results be discarded in two cases. Of the other seven, two outliers were discarded, and in the remaining five results were acceptable in the wider range which resulted.

To determine reproducibility in laboratory performance slides have been resubmitted to individual laboratories. The entire Survey C-15, was devoted to a study of reproducibility as is shown in Table III. It can be seen that most laboratories tend to be precise or reproducible in their diagnoses with certain laboratories tending to make consistent errors in evaluation.

Atypical Cells in Pleural Fluid Characteristic of Systemic Lupus Erythematosus

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Pleuritis and pleural effusion are common symptoms of systemic lupus erythematosus and may be among the earliest manifestations of this disease.[7] Until multisystem involvement becomes evident and the more commonly recognized clinical features of systemic lupus erythematosus appear the underlying disease responsible for the pleural effusion may remain undiagnosed. Examination of pleural fluid from ten patients with systemic lupus erythematosus has revealed atypical cells which appear to be characteristic of this disease. This report concerns the nature and derivation of these atypical cells as determined by a correlative study utilizing cytologic and histologic techniques and employing light, fluorescent, and electron microscopy

Materials and Methods

Ten patients were selected for this study on the basis of pleural effusion, lupus erythematosus cells in peripheral blood, and characteristic clinical and/or pathologic evidence of multisystem involvement. All were women The average age was 41 years. The duration of symptoms varied from few days to several years. Pleural fluid was examined in standard fashion utilizing centrifugation and microfiltration techniques, Papanicolaou stains and light microscopy In selected cases special stains (Wright, Feulgen, methyl green, pyronin, and periodic acid-Schiff) fluorescent microscopy (acridine orange) cell blocks, pleural biopsies, and electron microscopy were utilized. A control group consisted of pleural fluid from female patients in the same age range with pleural effusions of diseases other than systemic lupus erythematosus.

Results

Standard cytologic preparations stained by modified Papanicolaou technique revealed atypical cells scattered through an exudate consisting of mixed inflammatory cells and proteinaceous debris composed of eosinophilic, flocculent and fibrillar material (Fig 1) Atypical cells (Fig 2) had large, usually single, round or ovoid, hyperchromatic, eccentric nuclei coarse chromatin distinct, thin, regular nuclear membranes prominent nucleoli, usually single, and abundant cytoplasm in which condensation of opaque amphophilic, homogeneous material, sometimes showing vacuolar and globular changes, was visible often surrounded by clear zone beneath a distinct cell membrane. The exudate mainly consisted of lymphocytes, histiocytes, plasma cells, and neutrophils in varying proportions. Most fluids contained erythrocytes. Mesothelial cells were numerous in some fluids, rare in others. Typical lupus erythematosus cells were not seen in eight of the ten specimens.

Wright stains in selected cases demonstrated the hyperchromatic nuclei and amphophilic cytoplasm of the atypical cells. A semilunar clearing of cytoplasm adjacent to the eccentric nucleus was visible in some of these cells, and their resemblance to plasma cells was noted Typical lupus erythematosus cells could not be seen in the exudate

Feulgen stains did not reveal DNA in the cytoplasm of the atypical cells

Acridine orange stains under fluorescent microscopy showed the characteristic red fluorescence of RNA in the cytoplasm of these cells. Diffuse green fluorescence w noted in the proteinaceous background material.

Periodic acid-Schiff stains of cytologic prepara

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Reprint equests should be sent to Miss Kelley Cytotechnologist, Dept of Pathology Grady Memorial Hospital, 80 Butler Street, S. E., Atlanta, Georgia 30303 cytology the requirements are not stringent. In exfoliative cytology the laboratory director or supervisor or cytotechnologist must rescreen for proper staining and correct interpretation at least a ten percent random sample of gynecological smears which have been interpreted to be in one of the benign categories by personnel not possessing director or supervisor qualifications All gynecological smears interpreted to be suspicious or positive must be confirmed by the laboratory director or qualified supervisor as must all non-gynecological preparations, whether positive or negative. *There must be quality control for non-manual* methods similar to that provided in other non-manual laboratory procedures. All smears shall be retained for not less than two years from the date of examination.

These requirements of the Clinical Laboratories Improvement Act establish minimal internal quality control standards for a diagnostic cytology laboratory While one might suggest that other forms of quality control might be equivalent or superior at least the practice of rescreening is defined The stipulation of a ten percent random rescreen of gynecological material and a complete rescreen of all other material should assure that *cytotechnologists will be motivated if only to* avoid being found in error The retention of slides will help particularly in establishing the laboratory s false negative rate The danger in defining quality control in such regulations is that it may give some directors the impression that these standards represent a total quality control program, with no deviations possible Certainly nothing can be further from the truth. The regulations do not spell out in detail quality control aspects of staining record management, or clinical correlation More sophisticated systems have been developed to measure cytotechnologist reproducibility accuracy and workload.[1] The interpretation of sputum cytology slides has been shown to vary considerably from screener to screener and from cytology center to cytology center Surveys of *cytopathologists have also shown* lack of concordance in the diagnosis of lesions of the uterine cervix.[11] Internal quality control can serve an important purpose *to decrease the degree of* discordance between screeners in one cytology center

Interstate licensure also includes a requirement for proficiency testing in exfoliative cytology Performance evaluation as a technique is still in its infancy but experiences gathered from state and national programs have provided a starting point for further growth [4 6 7 8]

SUMMARY

The Wisconsin program is the first attempt to develop an external evaluation system for cytology As other programs emerge critical comparison of their methodology *and results will enable* concerned directors to design improved systems for overcoming problems of interlaboratory variability accuracy and reproducibility in the field of diagnostic cytology

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homogeneous, amphophilic cytoplasm (Fig. 3) Finer details of these cells were better visualized in cytologic preparations A tendency to cluster in rosette-like formations with globular expansion and hyalinization of their cytoplasm was noted. Typical lupus erythematosus cells were not evident. The remainder of the exudate was mixture of inflammatory cells, erythrocytes and proteinaceous debris.

Sections of pleura stained with hematoxylin and eosin and examined by light microscopy revealed alterations characteristic of systemic lupus erythematosus. Areas involved by pleuritis showed replacement of the mesothelium by fibrinous exudate filled with degenerating inflammatory cells and cellular debris (Fig. 4) In the underlying fibrous tissue of the pleura collagen necrosis, fibrinoid material, hematoxylin-staining bodies, smudging of mesenchymal cell nuclei, fibroblastic activity and mixed inflammatory infiltrate could be seen Typical lupus erythematosus cells could not be found, but large atypical cells with hyperchromatic, eccentric nuclei, prominent nucleoli, and abundant, amphophilic, homogeneous cytoplasm appeared as part of the inflammatory reaction in the fibrous tissue of the pleura (Fig 5) Although their nuclei and cell bodies were distorted by surrounding tissue these cells bore striking resemblance to the cells found in the pleural fluid, demonstrated similar staining properties, and seemed to be gaining access to the pleural fluid through the altered pleural surfaces

Electron microscopic examination of pleural tissue revealed large atypical cells scattered through the inflammatory infiltrate (Fig 6) Many had undergone degenerative changes making them unrecognizable but those better preserved showed nuclear and cytoplasmic characteristics of plasma cells[24] The nuclei were round or ovoid and eccentric. The chromatin was arranged in coarse clumps. Nucleoli were prominent. The cytoplasm was abundant and contained granular endoplasmic reticulum in winding pattern, mitochondria, irregular dense structures, and vacuoles (Fig. 7) Fibrinous exudate, macrophages, mixture of inflammatory cells, and altered collagenous tissue constituted the other components of the exudate

DISCUSSION

Pleural effusion without an obvious cause such as congestive heart failure pulmonary infiltrate renal or hepatic disease trauma or inflammation affecting pleural surfaces, pulmonary infarction or neoplasm has, traditionally been considered indicative of tuberculous infection until proved otherwise especially when associated with positive tuberculin test Viral diseases are currently encountered with increased frequency in this syndrome. When the usual infectious, hemodynamic, metabolic, nutritional, traumatic, and neoplastic mechanisms for pleural effusion cannot be documented consideration should be given to collagen diseases, particularly systemic lupus erythematosus, to explain an otherwise "idiopathic pleural effusion Systemic lupus erythematosus is no longer regarded as rare, especially in women of childbearing age[11]

Pleuropulmonary involvement is common in systemic lupus erythematosus[18] It is often accompanied by pleural effusion and sometimes precedes the appearance of other signs of the disease. A pleural effusion discovered before multisystem involvement is apparent may for while, be labeled idiopathic" if commonly considered causes are absent. Most patients with pleural effusion due to untreated systemic lupus erythematosus would be expected to show some laboratory evidence, i.e. positive lupus erythematosus cell preparation, antinuclear antibodies and other immunologic abnormalities, serum protein alterations, and hematologic changes Lupus erythematosus cells have been described in pleural fluid and pleural biopsy tissue, but were not found in the pleural fluids in this study Atypical cells of mesenchymal derivation with features of plasma cells appeared characteristic of this disease, scattered through fibrinous exudate, among mixed inflammatory cells These cells were not found in pleural fluid of other diseases except in case of malignant hypertension on hydralazine

FIG. 5 Atypical cells in pleural inflammatory infiltrate (Hematoxylin and eosin × 450).

tions revealed PAS-positive material in the cytoplasm of the large atypical cells.

Methyl green pyronin stains of similar preparations demonstrated red staining of the cytoplasm of these cells.

Cell blocks prepared from pleural fluid, sectioned, and stained with hematoxylin and eosin revealed many large, atypical cells with round or ovoid, hyperchromatic nuclei and opacified,

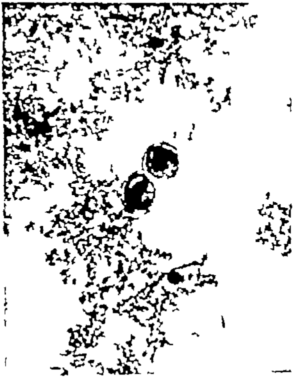

FIG 1 Large atypical cells and lymphocytes in proteinaceous debris (Papanicolaou × 100)

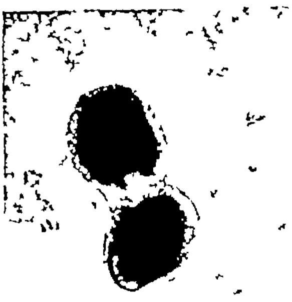

FIG 2 Atypical cells. (Papanicolaou × 450)

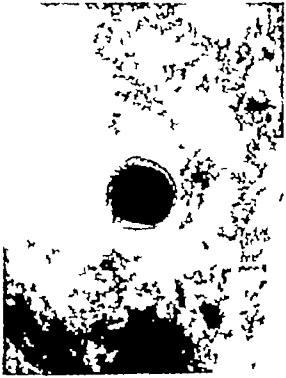

FIG 3 Atypical cells in cell block. (Hematoxylin and eosin × 450)

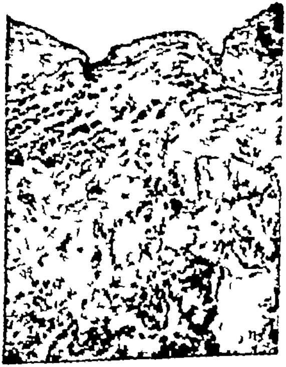

FIG 4 Pleural surface with inflammatory exudate. (Hematoxylin and eosin × 100)

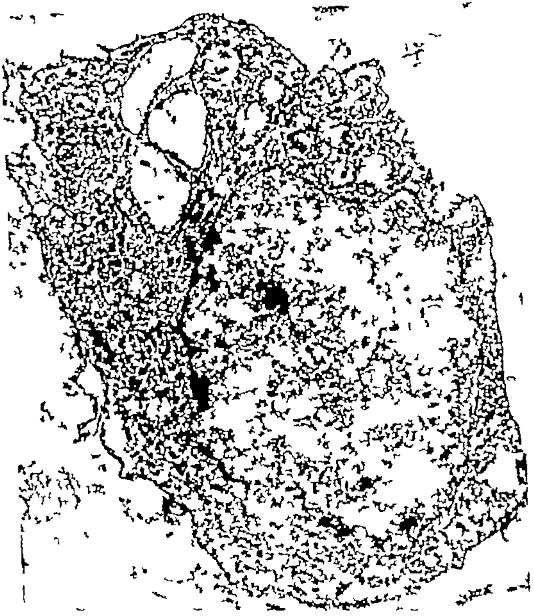

Fig. 7. Atypical cell (× 25,500)

Summary

Pleural effusion of undetermined cause is of particular importance in young and middle aged women since it may occur as the first manifestation of systemic lupus erythematosus. Because this disease is no longer regarded as rare, it should be considered in the diagnostic evaluation of pleural effusions. Atypical cells observed in cytologic preparations appear to be derived from the inflammatory infiltrate of the pleura. These atypical cells, when studied by light, fluorescent, and electron microscopy, resembled plasma cells. Combined with other inflammatory cells, fibrinoid debris, erythrocytes, and scarce mesothelial cells in a fluid free of microorganisms or neoplastic cells they constituted a characteristic pattern observed in pleural fluids from eight of ten cases of systemic lupus erythematosus and only rarely

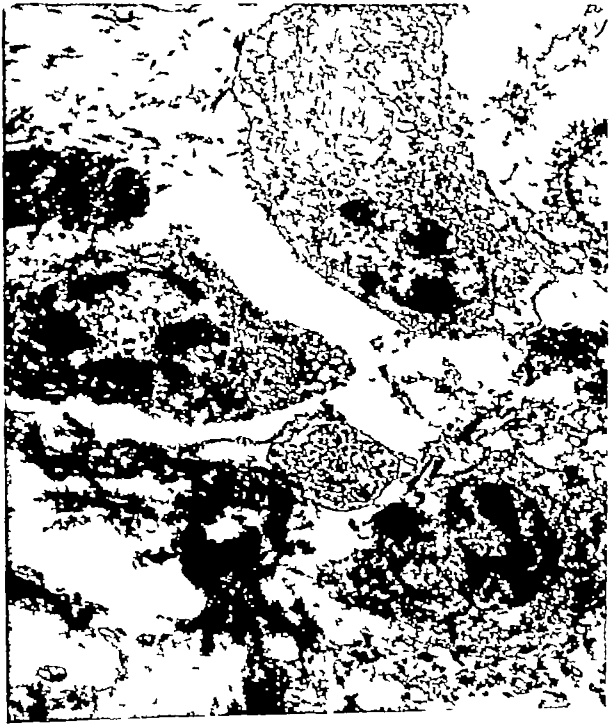

FIG. 6. Atypical cell with features of plasma cells (× 936)

therapy and in a case of leukemia, both conditions known to be associated occasionally with "false positive" lupus erythematosus cell tests and clinical manifestations resembling systemic lupus erythematosus. The variability of inflammatory cells was determined by the stage of the disease and presence or absence of secondary bacterial infection.[10, 11]

The atypical cells bore some resemblance to neoplastic plasma cells in multiple myeloma and have been mistaken for malignant cells in systemic lupus erythematosus.[1]

Sections of pericardium, peritoneum, synovial membranes, leptomeninges, choroid plexuses and kidneys revealed similar atypical cells scattered in areas of inflammatory reaction. There is anatomic evidence that access to body fluids associated with these structures could be gained by similar atypical cells.

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Observations of Eosinophilic Extracytoplasmic Processes in Pulmonary Macrophages

Progress Report

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The purpose of this paper is to report the observations of bipolar and other unusual forms of extracytoplasmic processes in pulmonary macrophages. These processes were observed by one of us† as an incidental finding while screening the sputum of persons enrolled in a research program in which chronic cigarette smokers were studied to evaluate the accuracy of detecting early occult carcinoma. In our search through the literature, we could find no evidence of these macrophages having been described.

MATERIALS AND METHODS

In this study sputum specimens from 909 smokers were examined and compared with findings in a non-smoking control group. Spontaneous and induced bronchial mucus specimens were obtained as necessary. A cough was induced in patients who could not expectorate a satisfactory sample by having the patient breathe mist of sterile distilled water from a DeVilbiss Ultrasonic Nebulizer Model 800/883. A specimen was collected on three consecutive days. Each sputum was collected in a Petri dish, then streaked on four glass slides. The slides were put into Coplin jars containing equal parts of ether and 95 per cent ethyl alcohol. The staining of slides was done by the standard Papanicolaou procedure.

RESULTS

Of the 909 smokers examined, 381 displayed cells with conspicuous eosinophilic processes. Although these cells are found in 41.9 per cent of the enrollees studied, they constitute an infrequent occurrence: only a few cells found in a total specimen prepared on 12 slides per enrollee show these morphologic features. These cells have been observed in specimens produced both spontaneously and induced. Further investigation revealed that these same processes are present in some sputum specimens of a non-smoking control group, not included in these percentages.

The morphology and staining of these cells in bronchial mucus are very striking. The eosinophilic staining projections appear in axial positions on opposite ends of the macrophages. Eosin is consistently taken up by the processes in sharp contrast to the cyanophilic cytoplasm. Typical examples of these bipolar histiocytes are shown (Figs. 1 and 2). The projections range in size and shape from small beads to large globules or long thin bars or hooks.

Most macrophages have two processes directly opposite each other; however, a small minority have only one. Others have multiple processes with at least two projections formed directly opposite each other.

The cells in which these processes are found include the morphologic varieties of macrophages. They are found on cells with one to four nuclei, as well as cells containing brown or black pigment. The cytoplasm stains in the typical cyanophilic or amphophilic color.

This rare form of histiocyte has also been found in frozen pulmonary tissue sections in our laboratory.

DISCUSSION

The theory that alveolar macrophages are derived from alveolar tissue may be an explanation for these processes. They may represent a remnant of cytoplasmic processes from alveolar wall tissue. Papanicolaou demonstrated that endometrial cells have the apparent ability to differentiate into free phagocytes when exfoliated during the late menstrual and early proliferative stage of the menstrual cycle. A similar phenomenon may occur in the alveolar spaces. Pinkett, *et al* concluded from their investigation that approximately one-third of the alveolar macrophages are of alveolar origin and about two-thirds originate from the hematopoietic system.

Other hypotheses should be considered, including the possibility that these processes may result observed in other circumstances. Studies of pleural tissue indicate a mesenchymal derivation of these atypical cells and supported the diagnosis of systemic lupus erythematosus. These findings may be of diagnostic value especially when the patient is a female of childbearing age, and should lead to a search for other clinical and laboratory signs.

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ACTA CYTOLOGICA

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Light and Electron Microscopic Observations on Malignant Cells in Cerebrospinal Fluid from Metastatic Alveolar Cell Carcinoma

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The purpose of this paper is to report on techniques and results in which the light microscope and the electron microscope were used jointly to study malignant cells in cerebrospinal fluid On initial cytopathologic examination of these cells, they were interpreted as being derived from malignant neoplasm of undetermined type. Autopsy revealed alveolar cell carcinoma. Electron microscopic study of these cells showed ultrastructural patterns compatible with those of type II alveolar cells.

MATERIALS AND METHODS

All cells and tissues on which this report is based were taken from one patient whose clinical course and autopsy findings are summarized

Case History

The patient was 24-year-old white married female nurse who was admitted to her local hospital nine months prior to her death because of weakness of her left arm, left leg, and left side of the mouth Roentgenograms of the skull and chest, pneumoencephalogram, and brain scan were normal

After discharge from the hospital she began to have intense headache with severe vomiting Treatment with parenteral steroids and ACTH achieved dramatic improvement within few hours Several months later she was re-admitted because of headaches and vomiting Spinal fluid submitted for cytologic study showed large, bizarre cells, the nature of which was unknown. They were not thought to be malignant

She was referred to Duke University Medical Center for further evaluation 5 ½ months prior to her death A general course of diagnostic procedures was repeated with normal brain scans, lumbosacral spine films, and skull films. A chest film showing density involving the left lower lobe was interpreted as bronchopneumonia Four spinal fluid specimens were evaluated cytologically and each was considered to be diagnostic for malignant cells of undetermined type Spinal fluid was also submitted for electron microscopic studies.

The final hospitalization of this patient occurred at her local hospital five weeks prior to her death Her main complaint on admission was severe headaches Although these responded somewhat to medication the general condition of the patient gradually deteriorated. In her last few days she did not respond to any stimuli or any medication.

Autopsy Findings

Gross examination revealed an infiltrating neoplasm of the lower lobe of the left lung with metastases to upper lobe of left lung all lobes of right lung hilar and retroperitoneal lymph nodes, ovaries, breasts, bone pituitary gland and central nervous system

Microscopic examination of the primary tumor in the lung showed an alveolar cell carcinoma which generally was well differentiated. It was composed of alveolar septa lined by single layer of tall columnar cells with abundant, faintly basophilic, granular cytoplasm (Figs 1 and 2) Luminal structures suggestive of terminal bars and cilia were occasionally encountered Examination of such areas under oil succeeded for the most part in convincing us that these were either mucous strands or brush borders. The nuclei were basally placed, large round, and vesicular Usually one large nucleolus was present. Mitoses were rare In some areas, the tumor was less well differentiated and characterized by piling up of cells, papillary processes, and pleomorphism with bizarre giant cells. The metastases lacked the marked degree of differentiation of the primary tumor and consisted of fields of tumor cells occurring singly and without attachment to one another In the central nervous system there w

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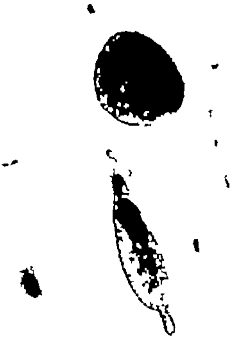

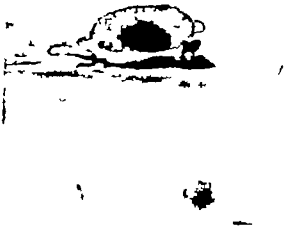

Fig.

Figs. 1 and 2. Unusual macrophages exhibit bipolar extracytoplasmic processes. These eosinophilic projections are strikingly dissimilar to the cyanophilic cytoplasm. Note anthracotic pigment phagocytized by cell in Fig. 2. Mag. × 1000

from excreted substances at bipolar orifices of the cells.

Because these processes are consistently bipolar and separated by 180° suggests an organized developmental phenomenon of the cell. The differential staining between the processes and the cytoplasm indicates a difference in density or chemical structure of the protein. This also suggests specific cellular organization which forms the processes.

To determine the specific nature, function and infrequent occurrence of these processes requires further collection of precise data.

SUMMARY

The occurrence of a rare morphologic form of pulmonary macrophage is reported. The nucleus and cytoplasm of these histiocytes are normal in appearance but eosinophilic extracytoplasmic processes present sharp contrast to the cyanophilic cytoplasm. The differential staining and morphology of these cells raise questions as to *their origin and possible function*. We are continuing our investigation.

A discussion as to the possible genesis of these projections is presented. These cells have been observed in sputum specimens from smokers and non-smokers, as well as in frozen sections of pulmonary tissue.

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F 3. Cerebral cortex. There is extensiv in ol ment of the subarachnoid space by metastatic al eolar cell carcinoma. Note poor differentiation of tumor as compared with the primary. Hematoxylin and eosin stain, ×250

ure from 20 to 30 microns. A characteristic cell had nucleus eccentrically placed in finely granular greenish-blue cytoplasm. The nucleus occupied one-half to two-thirds the diameter of the cell. Although the nuclear outlines remained deceivingly round and regular the nuclear contents were consistently markedly abnormal. Each nucleus contained one or more red macronucleoli some of which were irregular and angular. Chromatin distribution remained uniform and aried from powdery to coarsely granular. Nuclear hyperchromasia was not feature of these cells. Binucleated and trinucleated cells were occasionally seen. All of the cells showed fuzziness of outline suggestiv of microvilli and many degenerative cytoplasmic blebs. The background was clean and free of any evidence of inflammation. Rarely a lymphocyte or neutrophil was seen. A few cells showed bubbly cytoplasm, but no single or multiple secretory-type vacuoles were seen. These cells were interpreted as being malignant. W ere uncertain of their origin. Their presence in great numbers suggested extensiv involvement of the leptomeninges.

Electron Microscopic Observations

Ultrastructurally the cells were characterized by prominent uniform microvilli on their surface (Figs. 5 to 7). Numerous mitochondria, well developed Golgi abundant rough surfaced endoplasmic reticulum and numerous dense lysosomes were present. The nuclei were large and nucleoli prominent. Multivesicular bodies in various stages of formation were seen. Several types of lysosomes were noted. Some contained uniform homogenous internal contents, others showed beginning parallel concentric lamellations. Some of the more electron-dense lysosomes were surrounded by multiple membranous entrappings. I some areas vacuoles which contained only small amount of stable material, possibly representing mucin granules, were seen (Fig. 7). Numerous polysomes both free in the cytoplasm and ttached to the endoplasmic reticulum were present. In one area heavily laden with polysomes, an annulate lamella was identified (Fig. 8). Dilatation of the cell sap was prominent in the peripheral portion of some cells. This cell sap swelling when severe resulted in bleb-like

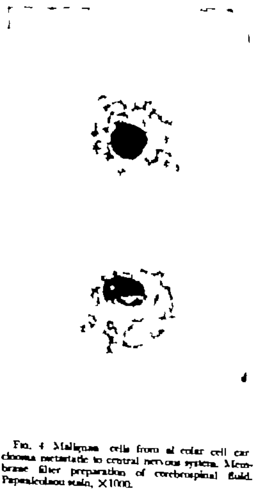

FIG. 4. Malignan cells from al eolar cell carcinoma metastatic to central nervous system. Membrane filter preparation of cerebrospinal fluid. Papanicolaou stain, ×1000.

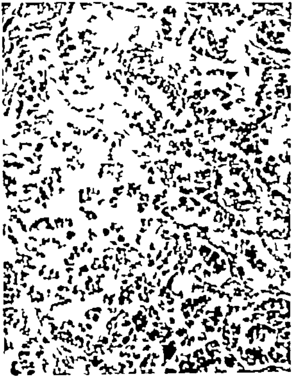

FIG 1 Left lower lobe of lung Alveolar cell carcinoma Hematoxylin and eosin stain, ×100.

extensive involvement of the leptomeninges (Fig 3)

Light and Electron Microscopy of Cells in Cerebrospinal Fluid

Four specimens of cerebrospinal fluid were taken for cytologic study The specimens varied in volume from ½ to 4 ml were clear and were fresh and unfixed when brought to the laboratory The fluids were filtered through membrane filters (Millipore S M 5 0 ± 1 2 47 mm) under a vacuum not exceeding 5 mm of mercury The filters were fixed in 95 ethyl alcohol and stained by the Papanicolaou method

For electron microscopy the cells were fixed in suspension by mixing equal volumes of cerebrospinal fluid and a solution which was prepared by mixing immediately prior to use equal volumes of 16% glutaraldehyde buffered with cacodylate and 4% osmium tetroxide buffered with s-collidine. Fixation was carried out for one hour at 4°C. Then the cells were filtered through membrane filters (Millipore S M 5 0 ± 1 2 47 mm) forming an incomplete monolayer After dehydration with a graded series of alcohols, propylene oxide was used to dissolve the membrane filter and replace the ethanol The cells were then embedded in a 0 5 mm layer of Epon by overlaying them with the unpolymerized resin and curing for 2 days at 56 C For sectioning small rectangles were cut from the disc and embedded in slots previously prepared in the tips of Epon blocks. Thick sections (0 5 – 1 0 μ) were examined with a light microscope and then thin sections were cut with a diamond knife mounted in an MT 1 Servall ultramicrotome The sections were doubly stained with lead citrate and uranyl acetate and examined with an Hitachi HS-7 HS-8 or HU-11E electron microscope

RESULTS

Light Microscopic Observations

A total of eight slides prepared from the filters was examined. A population of many similar abnormal cells was observed in all slides (Fig 4) There was only slight variation in cell number from slide to slide In each field examined under high dry lens (400 ×) four to seven abnormal cells were present The cells were moderately uniform in structure round to oval in shape and varied in

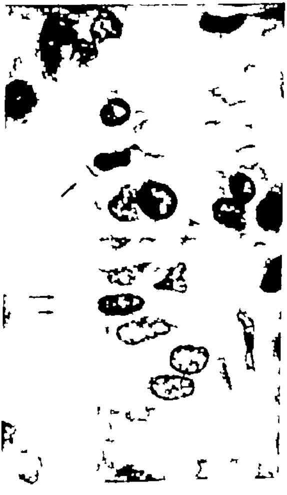

FIG 2. Same tissue as shown in Fig 1 at higher power to show striking degree of columnar differentiation in the neoplastic cells. Arrows point to luminal brush borders and inspissated mucous strands suggesting cilia. Hematoxylin and eosin stain, ×1000.

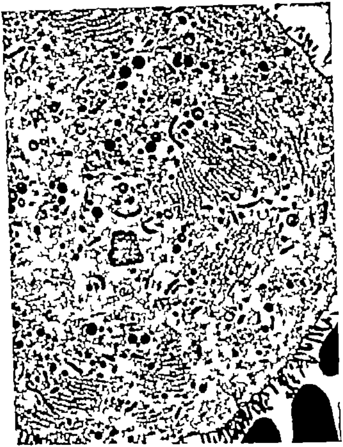

FIG. 6. This cell is characterized by the presence of microvilli and a well developed endoplasmic reticulum with abundant lysosomes. Surrounding the nucleus—which is cut tangentially—are numerous well-developed golgi. ×6800.

presence of ciliated tumor cells in one case. Studies by Spencer and Raeburn[14] and Fisher and Holley[4] each reported six cases of alveolar cell carcinoma with one case in each series described as showing ciliated cancer cells. The AFIP collection was later reviewed by Dr. John K. Frost who was unable to confirm the presence of cilia in any of the tumor cell. It was his conclusion that inspissated mucous strands were probably being mistaken for cilia. He has also reported this in other similar situations. In other tumors also, structures resembling cilia have been reported. Spriggs and Meek reported hairlike processes in some malignant cells in effusions. These were particularly striking in metastatic ovarian carcinoma. The electron microscope showed these to be cytoplasmic processes similar to elongated microvilli.

Annulate lamellae have been most carefully studied in invertebrate and vertebrate primitive germ cells but are being observed in a wider range of cell types now. We have been unable to find previous reports of annulate lamellae in alveolar cells—benign or malignant. Since the function of these bodies is not understood, the significance of such a finding in this patient will have to await more detailed understanding of subcellular structure and function.

SUMMARY

This paper reports the techniques and results from the use of electron microscopy to augment the routine cytopathologic study of cells in spinal fluid. The material for study consisted of multiple specimens of cerebrospinal fluid taken from a 24 year old white female. The patient later died after a nine month illness characterized by multiple bizarre neurologic symptoms. Light microscopy revealed a homogeneous population of cells, obviously malignant, but of uncertain derivation. Electron microscopic study showed prominent microvilli, numerous mitochondria,

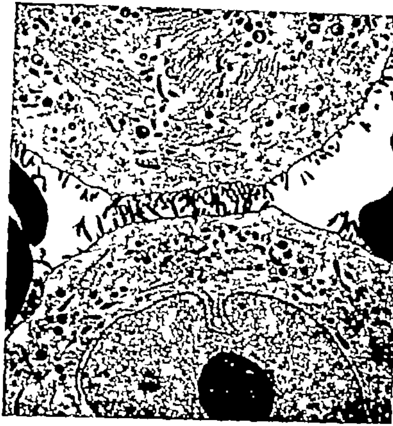

Fig 5 Numerous microvilli (MV) are present along the cell surfaces. Mitochondria (M) golgi (G) endoplasmic reticulum (ER) and numerous lysosomes (L) are seen. An *indented nucleus with prominent* nucleolus is present. ×5900.

formation at the periphery with loss of the micro*villous pattern (Fig 9)*

Discussion

Within the last 10 years, electron microscopic examination of normal lung tissue and of alveolar cell carcinoma has increased our understanding of the cell of origin of this neoplasm. Adamson and Senior in their study of biopsied tissue from a patient with alveolar cell carcinoma described the ultrastructure of the tumor cells.[1] The cytoplasm of the cells was densely packed with ribosomes, *mitochondria, and many osmiophilic* bodies producing an extremely dark cytoplasm. The ribosomes were *increased in number* and were free in the cytoplasm. Microvilli projected from the cell surfaces into the alveolar spaces. The nuclei were large, darkly stained and contained nucleoli. The nuclear membrane was noticeably invaginated and was indistinct. These investigators interpreted these cells as being similar in morphology to areas of normal type II alveolar epithelial cells which were also observed and described by them in their work. They described their normal type II alveolar epithelial cells as possessing microvilli, mitochondria, lamellar bodies, and nuclei with distinct nuclear mem*branes. In* another report lung tissues from three patients with alveolar cell carcinoma were studied by Coalson, Mohr, Pirtle, Dee and Rhoades.[4] Ultrastructurally the neoplastic cells in these cases were interpreted as being altered alveolar type II cells.

The term "type II alveolar cell" has been used to denote the large cuboidal cells located predominately within the angles formed by the junction of two or more alveolar septa. These cells are rich in organelles including ribosomes and mitochondria and have been *shown to contain* large lamellated osmiophilic bodies. These cells also have been *referred to as "granular pneumocytes."* They are to be differentiated from "type I alveolar *cells" also referred to as "membraneous pneumocytes."* These latter cells constitute the principle *lining* epithelium of the alveoli.[3]

In our study the ultrastructurally observed nuclear and cytoplasmic changed were similar to those previously cited in the literature for normal and altered type II alveolar cells. *The microvilli* are of particular interest when it is recalled that the controversy *over the existence of ciliated* cancer cells has involved observations on alveolar cell carcinomas as well as other neoplasms. In 1949 Swan published a review of nine cases of alveolar cell carcinoma drawn from the series of the Armed Forces Institute of Pathology.[14] He reported the

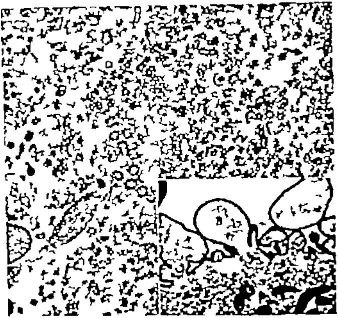

FIG. 8 An annulate lamella (AL) is present in an area exhibiting numerous free polysomes ×69,000

FIG. 9 (insert) Peripheral cytoplasmic blebs (B) are seen distorting and to some extent replacing the microvilli. However the intracellular organelles in the more central aspects of the cell appear normal and the cytoplasm there fails to reveal swelling ×14 000.

multiple foci of well-developed Golgi apparatus, several types of lysosomes, and one annulate lamella The ultrastructure of these cells resembled that which has been described for type II alveolar cells from the lung A topsy revealed alveolar cell carcinoma of the left lung with widespread metastases including massive involvement of the leptomeninges

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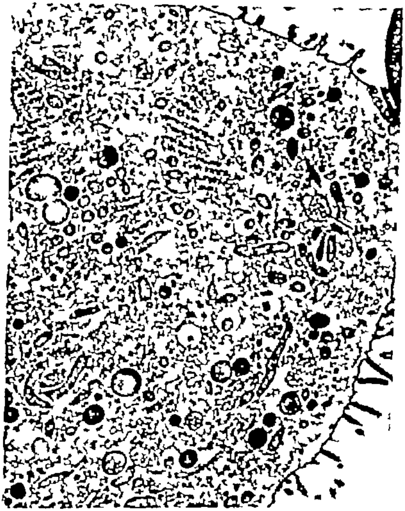
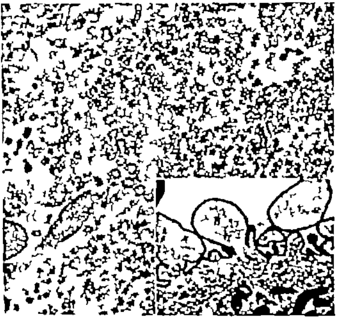


Fig. 7 The glycocalyx of the plasma membrane is apparent. Multivesicular (M B) bodies are seen and numerous lysosomes have patterns which vary from homogenous uniform density (L_1) and incomplete concentric lamellations (L_2) to multiple concentric lamellations (L_3) completely encircling the organelle. ×23,000



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The Evaluation of Sex Chromatin as a Prognostic Factor in Carcinoma of the Breast

A Preliminary Report

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Cytology Service, Department of Pathology, Memorial Hospital for Cancer and Allied Diseases, New York, New York 100?1, U.S.A.

The correlation between sex chromatin in tumor cells and clinical behavior of breast cancer was studied by a number of investigators. In most of the prior studies such evaluations were based on histologic material. Thus, Moore and Barr presented evidence that sex chromatin was present in a high percentage of nuclei in well-differentiated breast cancers and in a low percentage of nuclei in poorly differentiated or undifferentiated tumors[7]. Kimel and Shirley[3] suggested that response to therapy, particularly hormonal, was in proportion to the incidence of sex chromatin present in nuclei of tumor cells. Wacker and Miles demonstrated a direct favorable correlation between the percentage of tumor cells with sex chromatin and survival of patients. Similarly, Schiödt[8] found a somewhat higher 5 year survival rate in a small number of patients with over 30 per cent of sex-chromatin positive cancer cells.

Studies of sex chromatin in breast cancer cells in cell spreads were carried out principally in Europe[1,8]. Extensive work on correlation between sex chromatin, nuclear size, and DNA content was performed by Kallenberger and her group[4]. Kallenberger's conclusions were remarkable: in her material of 60 breast carcinomas, patients with sex chromatin positive tumor cells survived longer independently of tumor grade or the presence of axillary lymph node metastases at the time of surgery.

The purpose of the present study was to analyze in smears the correlation between the rate of sex chromatin in the nuclei of breast cancer cells with tumor recurrence, histologic grade and lymph node metastases.

MATERIAL AND METHODS

One hundred consecutive cases of female breast cancer were the subject of this investigation. At the time of frozen section direct smears were obtained by scraping the cut surface of tumors and spreading the material thinly on clean glass slides. The smears were fixed immediately in 95% ethyl alcohol. A modification of Guard's stain[2] was used according to the following procedure.

The slides were transferred from 95% alcohol to 70% to 50% and stained in Biebrich scarlet for 5 minutes. They were rinsed in 50% alcohol and stained in Fast Green FCF for two to four hours. Feulgen stain proved unsatisfactory on this material.

In each case 100 or more tumor cells were counted under oil immersion and the percentage with clear cut sex chromatin noted. Slides with less than 100 cells were rejected.

Very strict criteria for the identification of sex chromatin were maintained because of the irregular chromatin structure and prominent nucleoli of tumor cells: only those triangular or elliptical structures closely adhering to the nuclear membrane approximately 1 μ in diameter and red to muddy brown in color were counted (Figs. 1 and 2). The counts were repeated twice and spot checked by an independent observer. No attempt was made to separate the cases with two or more sex chromatin bodies per cell from those with only one body. In virtually every case there was a cancer cell or two with definite or probable double sex chromatin body. In some cases such cells were numerous.

All patients were uniformly treated by radical mastectomy with axillary lymph node dissection. There was no pre-or postoperative radiation. Correlation with histologic findings was carried out in all the cases. The histologic material comprised a minimum of two sections of the primary tumor, representative sections of the four quadrants of the breast and a section of the nipple. The axillary lymph nodes were identified according to three levels: level I being the most proximal and level III the most distant to the breast.

Classification and grading of tumors has been carried out according to the criteria long established at the Memorial Hospital, based on histologic patterns and cell size. Grade I cancers are the

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This work was supported in part by a grant from the Christine Sonntag Foundation.

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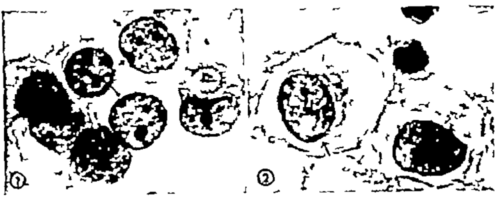

FIG 1 Breast cancer cells showing single and multiple sex chromatin bodies (arrows)
FIG 2 A large breast cancer cell with single sex chromatin body (arrow)

infrequent tubular carcinomas, grade II are the majority of duct cancers forming glandular tubular, or papillary structures Grade III are small celled, infiltrating (scirrhous) duct carcinomas or diffusely growing duct carcinomas showing absence of differentiation Intraductal carcinomas, lobular carcinomas in situ, infiltrating lobular carcinomas and medullary carcinomas with lymphoid stroma are not graded

In the present study there were no medullary carcinomas with lymphoid stroma with their uniquely favorable prognosis Cases of lobular carcinomas in situ were excluded Cases of infiltrating lobular carcinoma were classed together with grade III duct carcinomas to avoid unnecessary fractionation of the data

There were two cancers of the male breast during the period of investigation No definite sex chromatin bodies were observed in either case

Follow-up information ranging from one to five years was obtained from the hospital files and surgeons offices

RESULTS

The results are summarized in Table I It may be seen that 26 patients had counts of less than 10 per cent 47 patients had counts between 10 and 19 per cent and the remaining 27 patients had counts of over 20 per cent or better In the last group four patients had counts of 30 per cent or more

No recurrent cancer was observed so far among the 27 patients with sex chromatin counts of 20 per cent or more In the remaining 73 patients with counts below 20 per cent, 13 recurrences were noted (17.8%)

The relationship between the grade of tumor and the sex chromatin counts is explored in Table II It may be seen that the grade of tumor had no obvious bearing on sex chromatin counts The proportion of poorly differentiated high grade tumors with high and low sex chromatin counts was approximately the same.

TABLE I *Distribution of Sex Chromatin Bodies in Breast Cancer*

% of Sex Chromatin	No. of Cases		NED		Recurrence	
1–9	26	100%	19	82.2%	7	17.8%
10–19	47		41		6	
20–29	23	100%	23	100%	0	
30 & up	4		4		0	
Total	100		87		13	

NED = No evidence of disease from 1 to 5 years
Recurrence recurrent local or metastatic tumor within 1 to 5 years of follow-up

The relationship of sex chromatin counts with the presence of lymph node metastases at the time of original surgery was explored next With only a few exceptions the lymph node metastases were confined to level I As shown in Table III the distribution of lymph node metastases was approximately the same in patients with counts below 20 per cent and in those with counts above 20 per cent Yet there was striking difference in the rate of recurrent cancer between the two groups there were 10 recurrent cancers in 29 patients with positive nodes and counts below 20 per cent, no recurrences were observed so far among the 12 patients with positive nodes and sex chromatin counts of 20 per cent or better

DISCUSSION

The preliminary results strongly suggest that the presence of sex chromatin in cells of mammary carcinoma does not correlate well with histologic patterns as defined above This observation is in full accord with the view of Atkin I Kallen-

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Further Experience with Direct Vision Brushing Cytology of Malignant Tumors of Upper Gastrointestinal Tract Histopathologic Correlation with Biopsy*†

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Roentgenographic and endoscopic examinations are of great value in detecting cancer of the upper gastrointestinal tract. However they provide only macroscopic impressions on the nature of the lesion. The newer fiberoptic endoscopes have greatly facilitated sampling for biopsy and cytology under direct vision.

Our experience with the use of brushing cytology under direct vision was first reported in this Journal by Kobayashi, *et al* and brief report on the combined use of cytology and biopsy was reported elsewhere.[12] Since then, we have greatly enlarged our experience and a correlative study of the biopsy results has been completed. The purpose of this communication is to emphasize the important role and convenience of combining direct vision brushing cytology with biopsy to establish definitive diagnosis of cancer of the stomach and esophagus.

MATERIAL AND METHODS

Our technique of direct vision brushing cytology and biopsy has been previously reported.

The cytology smears are fixed in 95 ethyl alcohol, immediately after preparation, with the exception of two smears that are air dried for May-Gruenwald-Giemsa staining. In addition to the routine Papanicolaou staining method, two other staining methods are in use after alcoholic fixation: the acridine-orange fluorescence and the Shorr's polychromatic methods. In this communication are reporting only the results of the Papanicolaou method, because this is the only one used for routine diagnostic evaluation. The other mentioned methods, are still in experimental phase in our laboratory and will be the object of later separate report.

Using the above techniques, during the period from August 1968 to September 1970, we performed direct vision cytology in 269 patients and biopsy in 209 patients.

RESULTS

Cytology

A positive cytodiagnosis was obtained in 49, or 90.7 per cent of 54 proven malignant tumors. Three of 215 patients with benign conditions, or 1.4 per cent were falsely positive (Table I). Table II shows the results in the various types of tumors and the respective percentages of positive diagnosis.

Biopsy

Positive biopsy results were obtained in 43, or 81.1 per cent, of 53 malignant tumors (Table III). The various types of tumors biopsied are shown in Table IV.

Combined biopsy and cytology

In the 50 cases of malignant tumors of the upper gastrointestinal tract in which both procedures were performed, at least one was positive, and 35 both were positive (Table V). In 10 patients, cytology was positive while biopsy was negative. In 5 patients, biopsy was positive while cytology was negative. All cases were operated upon and the presence of malignant tumor was proven.

COMMENTS

The ultimate diagnosis of malignant tumor is based on histologic criteria. Therefore roentgenographic and endoscopic examinations are only indirect diagnostic procedures at a macroscopical level which can provide only presumptive diagnosis. On the other hand, cytology and biopsy yield definitive information at the microscopic levels, and these procedures are of special interest.

The present series is an enlarged one from the preliminary results reported previously[12] and it confirms all our conclusions and permits some more clarifications in their clinical usefulness and applications.

Accuracy

The diagnostic accuracy of our brushing cytology remained high, circa 90 per cent, comparable

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* This work was supported by the Gastrointestinal Research Foundation, Inc. of Chicago.

† Portions of this study were presented at the 18th Annual Meeting of the American Society of Cytology (Biltmore Hotel, Los Angeles, November 5, 1970).

TABLE II *Correlation Between Sex Chromatin Count and Grade of Tumor*

% of Sex Chromatin	Total	Intraductal Carcinoma	Tumor Grade		
			I	II	III Including Infil. Lobular Carcinoma
1–19	73 (100%)		1	57* (79%)	15† (17%)
20 & up	27 (100%)	0	0	19 (70%)	8 (30%)

Tumor grade: for explanation see text. Recurrence: see legend to Table I.

* 11 recurrences of tumor

† 2 recurrences of tumor

TABLE III *Correlation Between Sex Chromatin Counts & Lymph Node Metastases*

% of Sex Chromatin	Nodes Positive	Recurrence	Nodes negative	Recurrence
1–19	29/73 (40%)	10	44/73 (60%)	3
20 & up	12/27 (44%)	0	15/27 (56%)	0

Recurrence: for definition see legend to Table I.

berger's work, there was a positive correlation between small size of nuclei of cancer cells and diploid patterns of DNA with the presence of sex chromatin. However, Levy[6] pointed out that sex chromatin is more readily observed in its classical position in small nuclei and that its absence in large nuclei is probably artefactual. Thus neither growth pattern, degree of anaplasia nor size of nuclei adequately explain the presence or absence of the sex chromatin body in cancer cells.

Perhaps the most significant observation resulting from the present study pertains to the absence of correlation between sex chromatin counts and lymph node metastases at the time of original surgery. Approximately 40 per cent of all patients had lymph node metastases, usually to level I. However, subsequently there was a remarkable disparity of behavior between the group of patients with sex chromatin counts below 20 per cent and those with higher counts. One-third of the patients with low counts and lymph node metastases had recurrence of breast cancer, whereas none of the high count group had recurrent carcinoma after a similar span of observation.

These findings are in agreement with Kallenberger's observations and are rather puzzling. They apparently reflect some biologic differences between cancers or cancer bearers that are not readily explained on morphologic grounds alone and must be investigated further.

Because the relationship of various factors discussed in this paper may undergo a significant modification with the passage of time, the follow-up will be continued for a period of at least five years.

SUMMARY

Sex chromatin counts were performed on cancer cells in smears from 100 female breast cancers. The preliminary results suggest that neither the grade of tumor nor the presence of lymph node metastases at time of surgery have any direct correlation with the sex chromatin count. Of all the factors evaluated, sex chromatin counts of over 20 per cent appeared to have the greatest prognostic significance. None of the patients with breast cancer and sex chromatin counts of 20 per cent or greater had recurrence of disease, whereas 17.8 per cent of patients with counts below 20 per cent had recurrent cancer. The follow-up will continue.

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nosis of lesions detected by previous roentgenographic and endoscopic examinations. We consider the possibility of failing to detect a malignant tumor negligible due to strict adherence to this rule. We do not consider necessary saline washing cytology in a patient with normal roentgenographic study and also normal endoscopic visualization.

The respective roles of saline washing and direct vision cytology in large scale mass survey or in the follow-up of limited segments of the population, with high risk of developing carcinoma, such as patients with pernicious anemia, remain to be established.

False positive cytology

The problem of false positive cytology was commented upon in our previous communication. Since then we have had no other false positive cytodiagnosis, proving that those three errors resulted mainly from unfamiliarity with the material. The percentage of such errors has been then significantly lowered. It now represents only 1.4 per cent of the negative cases, or 6 per cent of the positive reports.

Safety

In agreement with other reports,[4-9] no complications have been encountered in our series. This is in contrast to the blind brushing and probing techniques, where significant accidents have been reported,[14, 15]

Clinical usefulness

Although some gastro-esophageal lesions are easily judged malignant by clinical and roentgenographic criteria, there remains a large group of cancers which appear benign, even after endoscopic inspection. In our experience, more than 15 per cent of gastric malignant lesions fall into this group. To help detect these lesions, many authors have emphasized the importance of periodic roentgenographic follow-up of all gastric ulcers to be certain that complete healing is achieved and maintained. Unfortunately even a malignant ulcer will sometimes heal, as shown by Palmer and Humphreys,[16] and Oanon, *et al.* To avoid delaying the treatment of gastric cancer some observers have suggested that all gastric ulcers be treated surgically, a course of action which we feel will lead to excessive and unnecessary morbidity and mortality. The adjunctive procedures discussed in this communication, brushing cytology and biopsy, offer the clinician additional help of considerable value in the accurate detection of those lesions with benign gross appearance.

However we would like to emphasize the complementary role of the techniques herein reported. In all cases, a radiographic study preceded the endoscopic examination. This sequence greatly facilitated identification of the lesions and planning the direct vision techniques. They do not replace radiographic or endoscopic visualization of lesions, but rather help in determining the precise nature of the lesions.

SUMMARY

From August 1968 to September 1970, direct vision brushing cytology was performed in 269 patients and biopsy in 209 patients with esophageal and gastric lesions.

A positive cytodiagnosis was obtained in 49 (90.7%) of 54 proven malignant tumors. Three of 215 patients with benign lesions were falsely positive. Positive biopsy results were obtained in 43 (81%) of 53 malignant tumors. In 50 patients with malignant tumors in which both procedures were performed, at least one was positive, and in 35 both were positive.

These findings confirm our preliminary results: high detection rate of malignant tumors, safety of the techniques, and excellent clinical usefulness in the differential diagnosis of upper gastrointestinal tract lesions.

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TABLE I *Diagnostic Accuracy of Gastro-Esophageal Brushing Cytology Under Direct Vision*

University of Chicago—August 1968 to September 1970 (269 patients)

	No. of patients	Positive cytology	Negative cytology	Positive percentage ± s. error
Cancer	54	49	5	90.7 ± 3.9%
Non-cancer	215	3	212	1.4 ± 0.8%

Papanicolaou staining method

TABLE II *Results of Direct Vision Brushing Cytology in Esophago-Gastric Malignant Tumors*

	No. of patients	Positive cytology	Negative cytology
Squamous cell-ca. esophagus	12	12	0
Adenocarcinoma cardia	10	8	2
Adenocarcinoma, stomach	28	26	2
Malignant lymphoma stomach	4	3	1
Total	54	49	5

Papanicolaou staining method.

TABLE III *Diagnostic Accuracy of Gastro-Esophageal Biopsy Under Direct Vision*

University of Chicago—August 1968 to September 1970 (209 patients)

	No. of patients	Positive biopsy		Negative biopsy
		Initial	Repeat	
Cancer	53	41	43 (81%)	10
Non-cancer	156	—	—	156

to the results reported by the Japanese investigators who employed similar methods.[1-4,6] The results of biopsy in our series are only slightly less accurate than those of Sakita and Takasu (85.7%)[7] and Kasugai, *et al.* (87.6%). The area most difficult to biopsy is the cardioesophageal junction; only 5 of 9 cases of malignant tumors in this area had positive biopsy results.

In our series, the combined use of cytology and biopsy, as seen in Table V, continued to provide a definitive pre-operative diagnosis in all cases in which both procedures were performed. 50 such cases now yielded the same 100 per cent detection rate noted in the preliminary series of 26 cases.[11]

TABLE IV *Direct Vision Biopsy in Esophago-Gastric Malignant Tumors*

(53 patients)

	No. of patients	Positive biopsy	Negative biopsy
Squamous cell ca., esophagus	12	10*	
Adenocarcinoma, cardia	9	5	4
Adenocarcinoma stomach	27	4	3
Malignant Lymphoma, stomach	4	4	—
Leiomyosarcoma, stomach	1	—	1
Total	53	43	10

* In two cases only the repeat biopsy was positive. The initial negative biopsy is not included in the table.

TABLE V *Diagnosis of Gastro-Esophageal Malignant Tumors by Combined Use of Biopsy and Cytology under Direct Vision*

(50 patients)

Cytology	Biopsy	No. of patients
Positive	Positive	35
Positive	Negative	10
Negative	Positive	3
Negative	Negative	0

Note: Table includes only initial procedures.

Indications for the procedures

Due to its previously mentioned advantages,[11] direct vision brushing cytology has practically replaced the saline washing method in our laboratory. This has resulted in substantial savings in time of the cytotechnicians, both in the collection of the material (they do not have to do it anymore) and in the screening of the stained smears. In our opinion, in a center equipped with an endoscopy clinic, saline washings of the esophagus and stomach should be reserved for those rare patients in whom there is contraindication for endoscopy. In our experience, the trainees in endoscopy are easily taught the techniques of brushing cytology and biopsy under direct vision, and as the availability and use of the modern fiberscopes increases, these techniques will become more and more routinely applied.

In our endoscopy clinic, the main indication for the direct vision techniques is the differential diag-

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Squamous Cell Changes Associated with the Presence of *Candida sp* in Cervical Vaginal Papanicolaou Smears

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The purpose of this preliminary report is to define squamous cell changes associated with the presence of *Candida sp* in cervical-vaginal smears. These changes are noted most frequently in smears with preponderance of intermediate squamous cells present

Histopathologic and cytologic changes previously have been noted in the lung [13, 14] The histopathology of candidiasis has been thoroughly documented Histologically abscess formation, fibrosis, polymorphonuclear leukocyte, lymphocyte and plasma cell infiltrations have been noted in association with *Candida sp* [8, 11, 13] Clinically the infection of the vaginal mucosa is characterized by irritation, pruritus and thin discharge

A useful adjunct to cytology is recognition of parasitic infections. Detection is facilitated by the fact that the infection often accompanies characteristic cytologic pattern which can be recognized even before the organism is seen on the smear Thus, while only the organism itself is pathognomonic for the disease, recognition of specific cytologic pattern associated with parasite can greatly increase speed and accuracy of its detection Detection of candidiasis has become more important because its incidence is increasing. This apparent increase has been correlated with better detection methods, increased use of antibiotic therapy [14, 15] and oral contraceptives (especially progestational hormones) [17] The presence of an intermediate cell pattern with the use of combined hormonal and progesterone therapy has been established [9] A high incidence of *Candida sp* in diabetes and pregnancy has also been noted.

MATERIALS AND METHODS

Three hundred fourteen cases positive for *Candida sp* previously routinely screened during a three-year period were reviewed by tw cytotechnologists. One slide per case was screened. Smears with trichomonads present and smears with trophied cells present were purposely omitted from this study No specific associated vaginal flora were identified with the presence or absence of *Candida sp* on the smears. All slides had been stained with regressive four-minute hematoxylin Papanicolaou stain. The ages of all patients were noted if the information was available. Also noted was the squamous cell maturation level—5 per cent or more superficial cells present or fewer than 5 per cent superficial cells present. The smears with fewer than 5 per cent superficial cells present were considered to have an intermediate cell pattern.

The slides were considered in two groups one group of 265 cases had *Candida sp* hyphae and blastospores present, the other group of 49 cases had blastospores only present on the slides. Three hundred fourteen randomly selected smears with no hyphae or blastospores present were also reviewed as control group.

In the hyphae-blastospores group w found that 139 cases had all of our proposed changes present these cases were rescreened One hundred twenty-seven cases had an intermediate squamous cell pattern. W then subjected the 127 cases with an intermediate cell pattern to actual cell count One hundred cells per case were counted to determine the percentage of cells in one hundred cells with our proposed changes. A control group of 127 cases with an intermediate cell pattern and no hyphae or blastospores was similarly treated for control. The results of this study are tabulated in Tables I and II.

RESULTS

The squamous cell nuclear cytoplasmic, and/or pattern changes seen alone or in combination on the slides with *Candida sp* present were defined as follows.

Radial clumping (Fig 1): Squamous cells are situated radially along a linear axis with or without the presence of an obvious *Candida sp* hypha as that axis.

Vacuous cytoplasm (Figs. 2 and 3) Squamous cells with vacuous cytoplasm appear to have cytoplasmic areas devoid of stained material. The cytoplasm appears partially emptied. This change

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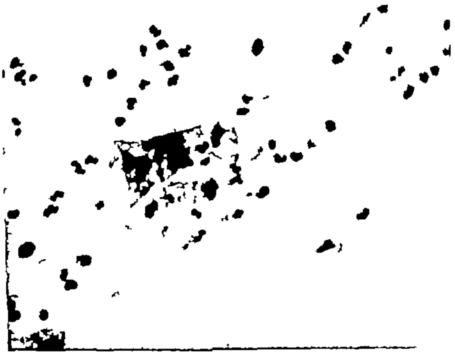


Fig 2 Squamous cell clump with acuous cytoplasm (×400)

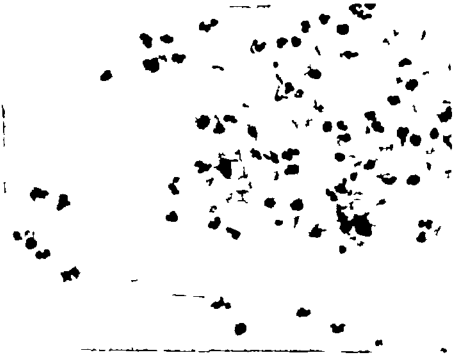


Fig 3. Single squamous cell with acuous cytoplasm. (×400)

TABLE I *Number of Cases with Changes Characteristic of Candida Sp*

Candida forms present	Number of cases	Mean age	Maturation		Epithelial characteristics—cases				
			Cases with 3% or more superficials	Cases with less than 3% superficials	Radial lumping	Vacuous cytoplasm	Nuclear change	Peri-nuclear haloing	Combined changes
Hyphae blastospores	263	33	73 28%	192 73%	173 66%	139 53%	176 66%	243 92%	139 53%
Blastospores only	49	46	34 69%	15 31%	3 6%	3 6%	11 22%	38 78%	4%
Control no hyphae or blastospores	314	4	155 49%	159 51%	11 4%	2 1%	36 12%	63 20%	0 —

TABLE II *Cell Types Present with Candida Sp*

Hyphae blastospore cases with combined changes	Cases with 3% or more superficial squamous cells	Cases with less than 3% superficial squamous cells	Total cells counted	Percent cells with epithelial characteristics per one hundred cells counted		
				Vacuous cytoplasm	Nuclear change	Peri-nuclear haloing
139	12 9%	127 91%	100 cells per case 137 cases	27%	56%	44%
Control no Hyphae-blastospores	0	127	100 cells per case 127 cases	0	2%	2%

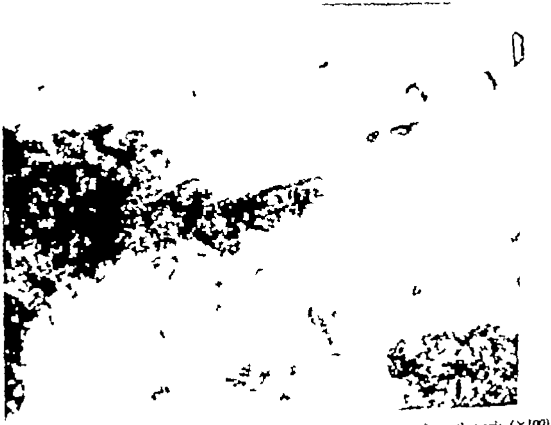


Fig 1 Squamous cells situated radially along the long axis with a *Candida sp* hypha as that axis. (×100)

Fig 2. Squamous cell clump with acuous cytoplasm (×400)

Fig 3. Single squamous cell with acuous cytoplasm (×400)

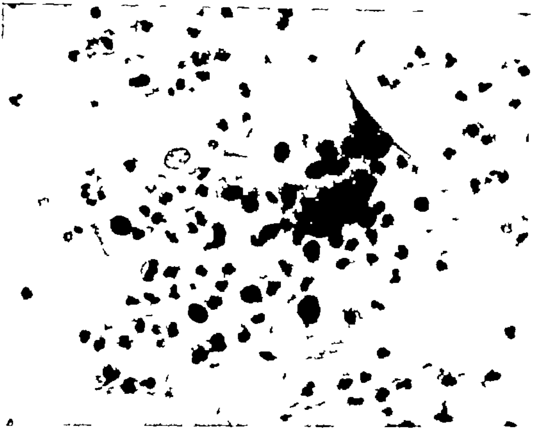


Fig 4 Intermediate squamous cell with increased nuclear activity Note increased chromatin deposit on the nuclear borders. (×400)

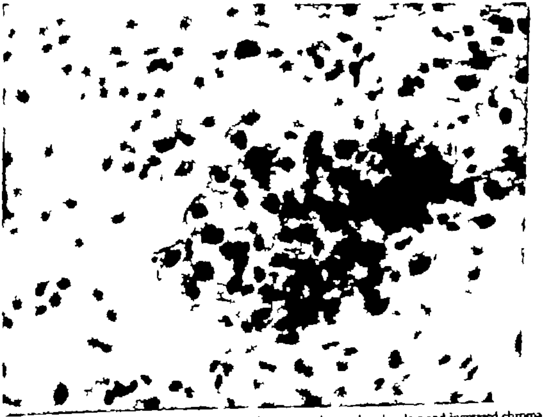


Fig 5 Squamous cells with increased nuclear ac[illegible] Note irregular nuclear borders and increased chromatin stain affinity (×400)

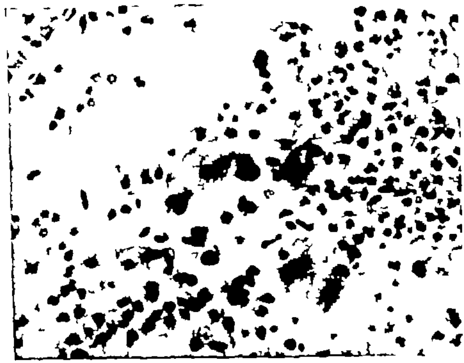


Fig 6. Perinuclear haloing Perinuclear haloing appears to enhance the increased nuclear activity (×400)

appeared to involve predominantly intermediate squamous cells Vacuous cytoplasmic changes were noted in singly exfoliated squamous cells and in clumps of squamous cells

Increased nuclear activity (Figs. 4 and 5) The nuclei of the intermediate squamous cells had slightly irregular nuclear borders, increased chromatin stain affinity nuclear enlargement inconsistent with the maturation of the cytoplasm and/or increased chromatin deposit on the nuclear borders Various combinations of the nuclear changes were seen, and varying degrees of severity of the individual changes were noted Perinuclear haloing appeared to enhance this change.

Perinuclear haloing (Fig 6) This cellular change was defined as an area of varying size and shape directly surrounding the nuclear border of squamous cell which is devoid of stained material. Relative size of halos varied from minimal or subtle haloing to accentuated, irregular peri-nuclear spaces

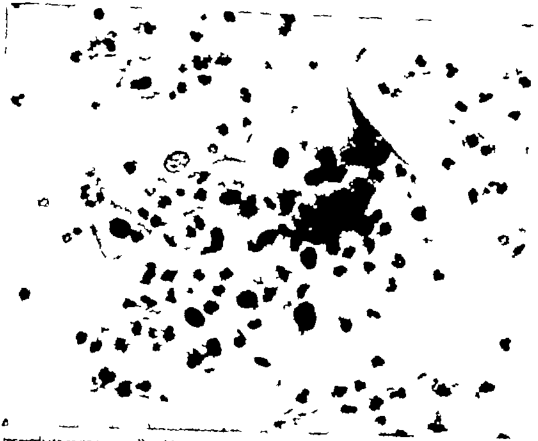
Combined changes Smears with all four proposed changes present—radial clumping, vacuous cytoplasm, increased nuclear activity and perinuclear haloing—were considered to have combined changes

DISCUSSION

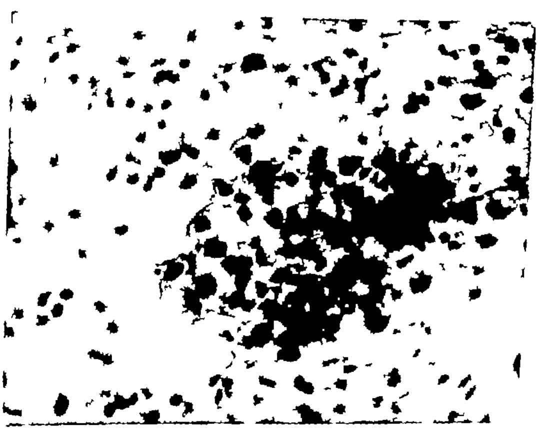
Table I shows that the women with a mean age of 46 usually had 5 per cent or more superficial cells present and blastospores only on the slides. The women with a mean age of 33 usually had fewer than 5 per cent superficial cells present (intermediate squamous cell pattern) and hyphae blastospores present on the slides The control group is evenly divided in maturation with mean age of 42 No conclusions can be reached from this information since menstrual data, presence or absence of pregnancy administered hormones or antibiotic therapy are unknown

There were fewer squamous cell changes associated with the presence of blastospores only and 5 per cent or more superficial cells present on the slides than were associated with the hyphae-blastospores intermediate pattern group The most significant finding is between the controls and the slides with hyphae-blastospores present Considerably fewer squamous cell changes are present when no *Candida sp* hyphae or blastospores are present

We rescreened the 159 cases which had radial clumping vacuous cytoplasm, increased nuclear activity and perinuclear haloing all present The results are shown in Table II One hundred



ig. 4. Intermediate squamous cells with increased nuclear activity. Note increased chromatin deposit on the nuclear borders. (×400)



5. Squamous cells with increased nuclear activity. Note irregular nuclear borders and increased chromatic stain affinity (×400)

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Exfoliative Cytology of the Urinary Bladder Irrigation Specimen

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The purpose of this paper is to present in detail new method for the cytologic detection of bladder cancer. This method utilizes a bladder irrigation specimen rather than voided or catheterized urine.

The principle of the bladder irrigation technique is similar to that of gastric cleansing and lavage.[illegible] In 1968 Villardel[illegible] reviewed the literature and concluded that the "highest percentage of accuracy (in the cytologic diagnosis of gastric carcinoma) is obtained using methods in which the stomach is aspirated, cleansed and vigorously washed. In the same article he emphasized the value of preparing paraffin blocks from centrifuged lavage material, since the blocks frequently contained fragments of tissue dislodged at the time of collection.

In the field of urinary cytology, Umiker[illegible] also stressed the value of the paraffin blocks by pointing out that neither a papilloma nor Grade I transitional cell carcinoma can be recognized on the basis of cell material alone.

In Umiker's review of prior literature,[illegible] he found the diagnostic accuracy of urinary cytology (using voided or catheterized urine) to vary from 76 to 100 per cent with generally converse false positive report rate of 1 to 12 per cent. More recently Kern reported diagnostic accuracy of 80 per cent accompanied by a false-positive rate of 20 per cent.

The high incidence of false-positive cytologic evaluations, using voided or catheterized urine, seems to accompany high degree of diagnostic accuracy. This, coupled with the urologist's opinion that he can visualize the majority of bladder cancers cystoscopically may account for the urologist's reluctance to rely on urinary cytology. However, it should be noted that there are numerous reports of cytologic detection of bladder cancer in patients whose cystoscopic examination failed to reveal the lesion.

Because the incidence of bladder cancer in the general population is low, most cytopathologists and urologists agree that cytologic examination of urine is not a suitable cancer screening procedure in patients who do not have urinary tract symptoms. However, our experience has proven that the bladder irrigation specimen is valuable adjunct to the cystoscopic examination of symptomatic patients, and cystoscopy has provided convenient means of applying the lavage technique to the urinary bladder.

This paper reports the details of benign and malignant cellular morphology of 335 urinary bladder irrigation samples, and describes the findings in carcinoma, atypia, and "normal" bladder irrigation specimens.

MATERIALS AND METHODS

Bladder irrigations were performed on 335 patients undergoing cystoscopic examination because of suspected or previously diagnosed urologic condition. Included were 176 males and 159 females referred to the Urology Services of both the Los Angeles County—University of Southern California Medical Center and the Center's outpatient clinic. Clinical histories of patients warranting such evaluation are shown in Table I.

Both catheterized urine and irrigation specimens were collected from each patient prior to cystoscopy. Catheterized urine was obtained at the time the obturator was removed from the sheath of the cystoscope. Irrigation specimens were obtained with the aid of Toomey syringe. Normal saline was agitated vigorously inside the bladder and 50 to 75 ml of irrigant was then withdrawn for cytologic study.

The urologists participating in this study were requested to obtain two irrigations if the first one collected was either grossly cloudy or bloody. It was felt that any tissue fragment dislodged from a neoplasm would appear in the initial return while repeat irrigation might contain less necrotic and inflammatory debris.

Both specimens were fixed immediately by doubling their respective volumes with 50% propyl alcohol. A duplicate report of the resident's cystoscopic examination accompanied each case to

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twenty-seven of those cases with combined changes had an intermediate squamous cell pattern. Considerably increased nuclear activity haloing and vacuous cytoplasmic change was present in the single cells counted in these 127 cases. The controls with an intermediate pattern and no *Candida sp.*, hyphae or blastospores present showed very little squamous cell change

Conclusion

The presence of *Candida sp* is often accompanied by a characteristic cytologic pattern. The ability to cytologically differentiate the pattern produced by *Candida sp* from other epithelial changes may save the patient repeat smears or indicate the necessity of follow-up studies. The purpose of this preliminary report is to define squamous cell changes associated with the presence of *Candida sp* in smears and to establish these changes as occurring more frequently in smears with predominantly intermediate squamous cells present. The following changes were found to be present radial clumping vacuous cytoplasm, increased nuclear *activity and peri-nuclear haloing*

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was scant or poorly preserved and cellular changes were inconclusive a report was sent out stating these findings and requesting further studies.

RESULTS

As an adjunct to cystoscopy cytologic evaluation of urinary bladder irrigations provided primary diagnostic information in six of the 20 cases of histologically proven carcinoma I three of these six cases the tumor was not seen at the first cystoscopic examination I the other three cases the tumor was seen by the cystoscopist, but the first biopsy did not demonstrate the lesion. The cytology in these six cases was diagnostic of malignancy

There were no false-positive diagnoses in this study There was one false negative diagnosis involving one of three low grade papillary transitional cell carcinomas. Cells from transitional cell papilloma tissue fragments, which appeared frequently in the cell blocks, made the definitive diagnosis possible in the other two cases. One of these three tumors was not seen cystoscopically

There were only 12 cases out of the total 333 in which definitive diagnosis could not be made.

Irrigations consistently yielded adequate, well preserved cellular material for microscopic evaluation I 287 of the 333 cases both catheterized urine and bladder irrigations were obtained from the same patient during cystoscopy When compared microscopically with the catheterized urine, the irrigation was characterized by:

) better preservation of cells

b) better cellular yield

c) better representation of bladder epithelium and

d) less contaminating background debris.

These differences were significant in the majority of cases (Figs. 1 and 2)

Proven Carcinomas (20 Cases, Table II)

Well preserved malignant cells were detected in the initial irrigation specimen obtained from each patient (Fig 3) I three cases no such cells were found in the catheterized urine from the patient or the cells found were too poorly preserved for evaluation (Fig 4) When malignant cells were present they predominated the cellular pattern. Eight of these cases will be described briefly

Cases 1 and 2 Both of these tumors were low grade papillary transitional cell carcinomas. A definitive diagnosis in both cases was made from tissue fragments which appeared in the cell block, not from the cellular pattern of the filter preparations. The slides in Case 1 had only predominance of small, benign columnar cells singly and in sheets. No tumor was visualized at cystoscopy I Case 2, cytology preparations revealed the

Fig 1 Catheterized urine Poorly preserved transitional cells. Compare with cells from bladder irrigation specimen of same patient in Fig 2 (× 500)

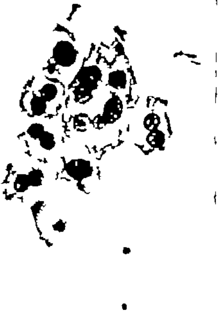

Fig 2 Bladder irrigation specimen Well preserved transitional cells. Compare with Fig 1 (× 500)

TABLE 1 *Bladder Irrigation Cytology Series Los Angeles County—University of Southern California Medical Center Clinical Histories—335 Patients*

Clinical history	Number of patients
Cancer of female genital tract	59
Hematuria	52
Bladder tumor follow-up	48
Benign prostatic hyperplasia	38
Renal pathology—TBC, pyelonephritis etc	23
Calculi	19
Cystitis	15
Urinary retention	10
Urethral stricture	9
Cancer of prostate	8
Obstruction—ureteral urethral bladder neck	7
Incontinence	5
Cancer of kidney	4
Neurogenic bladder	4
Squamous metaplasia bladder	4
Urinary tract infection	3
Diverticula	3
Trauma—gunshot wound automobile accident	2
Vesicosigmoid fistula	2
Hydrocele	1
History not stated	17
Total	335

the Cytology Laboratory. This allowed for comparison of the cytologic and cystoscopic findings. Whenever possible, visualized lesions were biopsied by the urologist.

For logistic reasons, several types of anesthetic jelly were in use by the Urology Department at the time this study began. The most suitable agent for purposes of cytology was the least viscous of these.*

Detailed procedures used by the Urology Service in the collection of specimens for cytology studies were as follows

Females The perineum was prepared with antibacterial detergent and rinsed with normal saline. A sterile cotton-tipped applicator coated with anesthetic jelly was inserted into the urethra, left for three to five minutes, and removed. The sheath of the Brown Buerger cystoscope along with its obturator was lubricated and introduced into the bladder. The obturator was removed and the bladder urine was emptied into sterile containers included in the surgical pack. One sample was for cytology studies and the other for routine bacterial culture and sensitivity. The bladder was then filled with 100 to 150 cc of normal saline and vigorously irrigated with a Toomey syringe.

Males. The perineum was prepared in the same way as described for females. Fifteen milliliters of anesthetic jelly was then introduced into the urethra and the penis was clamped for three to five minutes. After the clamp was removed, a well-lubricated scope was passed blindly or under direct vision into the bladder in the latter case, normal saline irrigant was flowing during passage. Collection of the catheterized and irrigated specimens was carried out by the procedure described for females.

The average volume of each specimen in this study was between 100 and 150 ml. At least half and sometimes all of this volume was utilized in preparing the cellular material for microscopic examination. These fluids were processed either by spreading centrifugate on glass slides or by collecting the cellular material on a membrane filter * in addition to the cell block. The gross appearance of the specimen determined which of these two methods was to be used

1 When the fluid was cloudy or turbid it was assumed that its centrifuged sediment would yield cellular material adequate for definitive diagnoses. This type of specimen was centrifuged at 1500 rpm for 15 minutes. To decrease the number of cells which might wash off during fixation and staining, slides were precoated with albumin. Prepared slides were fixed in a solution of Carbowax and 95% ethyl alcohol.

2. Fluids which were clear or only slightly cloudy in appearance were assumed to contain scant cellular material. These specimens were passed under vacuum through a 5.0 μ membrane filter a porous membrane which collected any cells suspended in the fluid on its surface. Prepared filters were fixed in 95% ethyl alcohol. The majority of our specimens were processed by the membrane filter technique. Both slides and filter were stained by the Papanicolaou method.

All excess centrifugate and any tissue fragments were made into cell blocks which were then sent to the Surgical Histopathology Laboratory for paraffin embedding and sectioning. Cell block sections were stained with hematoxylin and eosin.

The numerical Papanicolaou classification was not used in this study for reporting the cellular findings. When the cellular pattern was compatible with that of carcinoma, it was so stated. Cases in which the cellular pattern was not indicative of malignancy were signed out as "no malignant cells identified." In 12 cases where cellular material

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Anestacon,® from Conal Pharmaceuticals, Inc., Chicago, Illinois 60640, U.S.A.

Millipore Corporation, Bedford Massachusetts 01730, U.S.A.

Polyethylene glycol #1504 Union Carbide Corporation, South Charleston West Virginia 25303, U.S.A.

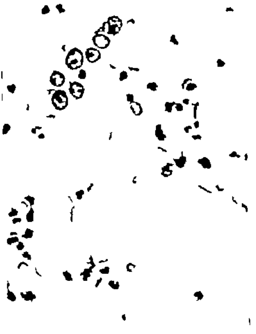


Fig 3 Bladder irrigation specimen Carcinoma cells from poorly differentiated transitional cell carcinoma (Case 9, Table II) Compare with cells from catheterized specimen in Fig 4 (× 500)

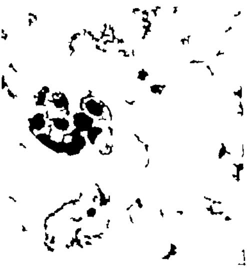


Fig 4 Catheterized urine specimen Poorly preserved transitional cells possibly from transitional cell carcinoma (Case 9 Table II). Cells are considered too poorly preserved to permit diagnosis. Compare cytology of this catheterized specimen with cytology of the bladder irrigation specimen in Fig 3 (× 500).

presence of few scattered groups of typical transitional cells with hyperchromatic nuclei The urologist' impression t cystoscopy was that the tumor had recurred.

Case 3 This patient' tumor was seen by the urologist. Histologic sections showed papillary transitional cell carcinoma, Grade I. W were unable to find any malignant cells in the catheterized urine specimen or the bladder irrigation. Irrigation material was scanty and no cell blocks were prepared in this case

Case 4 Because this patient did not tolerate cystoscopic examination without anesthesia, visualization of the bladder was not entirely satisfactory A trigonitis was seen, and it was believed to be the cause of the patient' symptoms. As result of the cytology report of cells present compatible in ppearance with origin from transitional cell carcinoma, the patient was anesthetized and a careful repeat cystoscopic examination was carried out. Trigonitis was still present and, in addition, two very small papillary lesions were identified at the dome of the bladder The entire area of involvement was estimated at 0 6 cm. Biopsy revealed one of the lesions as a papillary transitional cell carcinoma, Grade II. The second was so small that removal and fulguration resulted in its destruction and no tissue was available for microscopic examination

Case 5 In this case the urologist's impression t cystoscopy was "probable carcinoma of the prostate Cytologic evaluation of the smears and cell block preparations from the bladder irrigation indicated the presence of transitional cell carcinoma. The original histology slides were reviewed and cystoscopy was repeated. Again, no bladder lesion was identified. However tissue obtained by transurethral resection showed Grade IV transitional cell carcinoma (squamous carcinoma) invading the prostate. Subsequent review of the needle biopsy originally diagnosed as adenocarcinoma of the prostate revealed this diagnosis to be an error

Case 6. The bladder tumor in this patient was necrotic and encrusted with a crystalline deposit. In tissue sections of the first tw cup biopsies there was acute and chronic inflammation and necrosis. The third biopsy taken under anesthesia, was obtained by using resectoscope. Microscopic examination of this material revealed an anaplastic carcinoma presumed to be of transitional cell origin. The cytologic findings on the bladder irrigation preparations were reported as no malignant cells identified. Large numbers of inflammatory cells, *microcalculi*, and scattered atypical cells were also noted Two months after the original findings were reported, the cytologic material including the cell block and smears were reviewed and compared with the microscopic sections of the anaplastic tumor This review revealed malignant cells both

TABLE II. *Bladder Irrigation Cytology Series on 20 Cases of Carcinoma Proven by Tissue Section. Los Angeles County–University of Southern California Medical Center*

Case No.	Reason for Cystoscopic Examination (Clinical History)	Results of Cytologic Examination of U			Comments	Urologists' Impression at Time of Cystoscopy	Tissue Diagnosis
		Catheterized Specimen	Irrigation Specimen	Cell Block			
1	Follow-up exam of treated bladder tumor	Negative	Negative	Positive	Tissue fragment in cell block	No tumor seen	Papillary transitional cell carcinoma, Grade I
2	Follow-up exam of treated bladder tumor	No specimen	Negative	Positive	Tissue fragment in cell block	Recurrence of bladder tumor	Papillary transitional cell carcinoma, Grade II
3	Follow-up exam of treated bladder tumor	Negative	Negative	No specimen	None	Recurrence of bladder tumor	Papillary transitional cell carcinoma, Grade I
4	Hematuria	Positive	Positive	No specimen	None	No tumor seen; thyroid present postoperative	Papillary transitional cell carcinoma, Grade II
5	Benign prostatic hyperplasia	Positive	Positive	Positive	Tissue fragment in cell block	Carcinoma of prostate; no bladder tumor seen	1) Initial incorrect diagnosis on needle biopsy of prostate revised later on review to transitional carcinoma ducts of prostate 2) Transurethral biopsy showed transitional cell carcinoma of prostate
6	Diverticula	No specimen	Positive	Positive	Reported negative; reviewed positive; first biopsy negative	Area of induration and necrosis suspicious of tumor seen	Anaplastic carcinoma, presumed transitional cell
7	Follow-up exam of treated bladder tumor	Positive	Positive	No specimen	First biopsy showed chronic inflammation	Two small sessile lesions seen	Papillary transitional cell carcinoma, Grade III
8	Follow-up exam of treated bladder tumor	No specimen	Positive	Positive	First biopsy negative; tissue fragment in cell block	Recurrence of bladder tumor	Papillary transitional cell carcinoma, Grade II
9	Follow-up exam of treated bladder tumor	Negative	Positive	No specimen	None	Recurrence of bladder tumor	Poorly differentiated transitional cell carcinoma
10	Follow-up exam of treated bladder tumor	Negative	Positive	No specimen	None	Recurrence of bladder tumor	Transitional cell carcinoma, Grade II
11	Cervical cancer	Negative	Positive	Positive	None	Cancer of bladder invasion from uterine cervix	Squamous cell carcinoma, invaded bladder
12	Adenocarcinoma of prostate	Positive	Positive	No specimen	None	Obstructive cancer of prostate	Adenocarcinoma of prostate
13	Follow-up exam of treated bladder tumor	No specimen	Positive	Positive	None	Recurrence of bladder tumor	Papillary transitional cell carcinoma
14	Non functioning kidney hematuria	Positive	Positive	Positive	Bladder biopsy negative for malignancy	Possible bladder tumor	Carcinoma of cervix or ovary with ureteral involvement at autopsy
15	Hematuria	Positive	Positive	Positive	None	Tumor at bladder wall	Transitional cell carcinoma, Grade *II*
16	Hematuria	Positive	Positive	Positive	None	Tumor visualized	Transitional cell carcinoma, Grade *III*
17	Chronic cystitis	Positive	Positive	No specimen	None	Chronic cystitis in bladder tumor	Transitional cell carcinoma, Grade *III*
18	Hematuria	Positive	Positive	No specimen	None	Tumor localized	Transitional cell carcinoma, Grade *II*
19	Follow-up exam of treated bladder tumor	No specimen	Positive	No specimen	None	Recurrence of bladder tumor	Transitional cell carcinoma, Grade *II*
20	Hematuria	No specimen	Positive	Positive	Tissue fragment in cell block	Tumor visualized	Papillary transitional cell carcinoma, Grade II

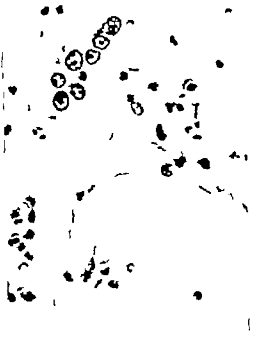


Fig 3 Bladder irrigation specimen Carcinoma cells from poorly differentiated transitional cell carcinoma (Case 8, Table II) Compare with cells from catheterized specimen in Fig 4 (× 500)

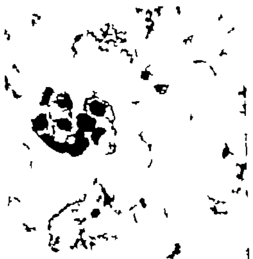


Fig 4 Catheterized urine specimen Poorly preserved transitional cells possibly from transitional cell carcinoma (Case 9, Table II). Cells are considered too poorly preserved to permit diagnosis Compare cytology of this catheterized specimen with cytology of the bladder irrigation specimen in Fig 3 (× 500).

presence of few scattered groups of atypical transitional cells with hyperchromatic nuclei. The urologist's impression at cystoscopy was that the tumor had recurred.

Case 3 This patient's tumor was seen by the urologist. Histologic sections showed a papillary transitional cell carcinoma, Grade I We were unable to find any malignant cells in the catheterized urine specimen or the bladder irrigation. Irrigation material was scanty and no cell blocks were prepared in this case

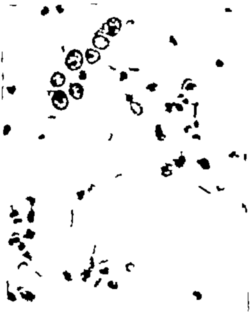
Case 4 Because this patient did not tolerate cystoscopic examination without anesthesia, visualization of the bladder was not entirely satisfactory A trigonitis was seen, and it was believed to be the cause of the patient's symptoms. As a result of the cytology report of cells present compatible in appearance with origin from transitional cell carcinoma, the patient was anesthetized and a careful repeat cystoscopic examination was carried out Trigonitis was still present and, in addition, two very small papillary lesions were identified at the dome of the bladder The entire area of involvement was estimated at 0 6 cm. Biopsy revealed one of the lesions as a papillary transitional cell carcinoma, Grade II. The second was so small that removal and fulguration resulted in its destruction and no tissue was available for microscopic examination.

Case 5 In this case the urologist's impression at cystoscopy was "probable carcinoma of the prostate. Cytologic evaluation of the smears and cell block preparations from the bladder irrigation indicated the presence of transitional cell carcinoma. The original histology slides were reviewed and cystoscopy was repeated. Again, no bladder lesion was identified. However tissue obtained by transurethral resection showed Grade IV transitional cell carcinoma (squamous carcinoma) invading the prostate. Subsequent review of the needle biopsy originally diagnosed as adenocarcinoma of the prostate revealed this diagnosis to be an error

Case 6 The bladder tumor in this patient was necrotic and encrusted with crystalline deposit. In tissue sections of the first two cup biopsies there was acute and chronic inflammation and necrosis. The third biopsy taken under anesthesia, was obtained by using a resectoscope. Microscopic examination of this material revealed an anaplastic carcinoma presumed to be of transitional cell origin The cytologic findings on the bladder irrigation preparations were reported as no malignant cells identified. Large numbers of inflammatory cells, microcalculi, and scattered atypical cells were also noted. Two months after the original findings were reported, the cytologic material including the cell block and smears were reviewed and compared with the microscopic sections of the anaplastic tumor This review revealed malignant cells both

TABLE II *Bladder Irrigation Cytology Series on 20 Cases of Carcinoma Proven by Tissue Section: Los Angeles County–University of Southern California Medical Center*

Case No.	Reason for Cytoscopic Examination (Clinical History)	Results of Cytologic Examination of Urine: Catheterized Specimen	Results of Cytologic Examination of Urine: Irrigation Specimen	Results of Cytologic Examination of Urine: Cell Block	Comments	Urologists' Impression at Time of Cystoscopy	Tissue Diagnosis
1	Follow-up exam of treated bladder tumor	Negative	Negative	Positive	Tissue fragments in cell block	No tumor seen	Papillary transitional cell carcinoma, Grade I
2	Follow-up exam of treated bladder tumor	No specimen	Negative	Positive	Tissue fragments in cell block	Recurrence of bladder tumor	Papillary transitional cell carcinoma, Grade II
3	Follow-up exam of treated bladder tumor	Negative	Negative	No specimen	None	Recurrence of bladder tumor	Papillary transitional cell carcinoma, Grade I
4	Hematuria	Positive	Positive	No specimen	None	No tumor seen, trigonitis—patient uncooperative	Papillary transitional cell carcinoma, Grade II
5	Benign prostatic hyperplasia	Positive	Positive	Positive	Tissue fragments in cell block	Carcinoma of prostate; no bladder tumor seen	1) Initial incorrect diagnosis on needle biopsy of prostate revised later on review to transitional carcinoma invasive of prostate 2) Transurethral biopsy showed transitional cell carcinoma of prostate
6	Diverticulum	No specimen	Pos	Positive	Reported negative; reviewed positive; first biopsy negative	Area of induration and necrosis suspicious of tumor seen	Anaplastic carcinoma, presumed transitional cell
7	Follow-up exam of treated bladder tumor	Positive	Positive	No specimen	First biopsy showed chronic inflammation	Two small sessile lesions seen	Papillary transitional cell carcinoma Grade III
8	Follow-up exam of treated bladder tumor	No specimen	Positive	Positive	First biopsy negative; tissue fragment in cell block	Recurrence of bladder tumor	Papillary transitional cell carcinoma, Grade II
9	Follow-up exam of treated bladder tumor	Negative	Positive	No specimen	None	Recurrence of bladder tumor	Poorly differentiated transitional cell carcinoma
10	Follow-up exam of treated bladder tumor	Negative	Positive	No specimen	None	Recurrence of bladder tumor	Transitional cell carcinoma Grade II
11	Cervical cancer	Negative	Positive	Positive	None	Cancer of bladder invaded from uterine cervix	Squamous cell carcinoma, invasion in bladder
12	Adenocarcinoma of prostate	Positive	Positive	No specimen	None	Obstructing cancer of prostate	Adenocarcinoma of prostate
13	Follow up exam of treated bladder tumor	No specimen	Positive	Positive	None	Recurrence of bladder tumor	Papillary transitional cell carcinoma
14	Non functioning kidney; hematuria	Positive	Positive	Positive	Bladder biopsy negative for malignancy	Possible bladder tumor	Carcinoma of cervix or ovary with ureteral involvement at autopsy
15	Hematuria	Positive	Positive	Positive	None	Tumor in bladder wall	Transitional cell carcinoma Grade II
16	Hematuria	Positive	Positive	Positive	None	Tumor visualized	Transitional cell carcinoma, Grade III
17	Chronic cystitis	Positive	Positive	No specimen	None	Chronic cystitis or bladder tumor	Transitional cell carcinoma, Grade III
18	Hematuria	Positive	Pos	No specimen	None	Tumor visualized	Transitional cell carcinoma, Grade II
19	Follow up exam of treated bladder tumor	No specimen	Positive	No specimen	None	Recurrence of bladder tumor	Transitional cell carcinoma, Grade II
20	Hematuria	No specimen	Pos	Pos	Tissue fragment in cell block	Tumor visualized	Papillary transitional cell carcinoma Grade II



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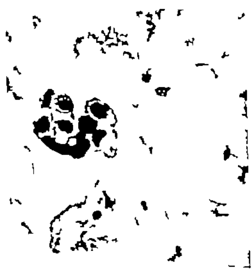

Fig 4 Catheterized urine specimen Poorly preserved transitional cells possibly from transitional cell carcinoma (Case 9, Table II). Cells are considered too poorly preserved to permit diagnosis Compare cytology of the catheterized specimen with cytology of the bladder irrigation specimen in Fig 3 (× 500).

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2	Follow-up exam of treated bladder tumor	No specimen	Negative	Positive	Tissue fragment in cell block	Recurrence of bladder tumor	Papillary transitional cell carcinoma Grade II
3	Follow-up exam of treated bladder tumor	Negative	Negative	No specimen	None	Recurrence of bladder tumor	Papillary transitional cell carcinoma, Grade I
4	Hematuria	Positive	Positive	No specimen	None	No tumor seen, trigonitis—p [illegible]	Papillary transitional cell carcinoma, Grade II
5	Benign prostatic hyperplasia	Positive	Positive	Positive	Tissue fragment in cell block	Carcinoma of prostate; no bladder tumor seen	1) Initial incorrect diagnosis on needle biopsy of prostate revised later on review to transitional carcinoma invasive of prostate. 2) Transurethral biopsy showed transitional cell carcinoma of prostate
6	Diverticula	No specimen	Positive	Positive	Reported negative, reviewed positive, first biopsy negative	Area of induration and necrosis suspicious of tumor seen	Anaplastic carcinoma, presumed transitional cell
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9	Follow-up exam of treated bladder tumor	Negative	Positive	No specimen	None	Recurrence of bladder tumor	Poorly differentiated transitional cell carcinoma
10	Follow-up exam of treated bladder tumor	Negative	Positive	No specimen	None	Recurrence of bladder tumor	Transitional cell carcinoma Grade II
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12	Adenocarcinoma of prostate	Positive	Positive	No specimen	None	Obstructive cancer of prostate	Adenocarcinoma of prostate
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14	Nonfunctioning kidney hematuria	Positive	Positive	Positive	Bladder biopsy negative for malignancy	Possible bladder tumor	Carcinoma of cervix or ovary with ureteral involvement at autopsy
15	Hematuria	Positive	Positive	Positive	None	Tumor at bladder wall	Transitional cell carcinoma, Grade II
16	Hematuria	Positive	Positive	Positive	None	Tumor localized	Transitional cell carcinoma, Grade III
17	Chronic cystitis	Positive	Positive	No specimen	None	Chronic cystitis, bladder tumor	Transitional cell carcinoma Grade III
18	Hematuria	Positive	Positive	No specimen	None	Tumor visualized	Transitional cell carcinoma Grade II
19	Follow-up exam of treated bladder tumor	No specimen	Positive	No specimen	None	Recurrence of bladder tumor	Transitional cell carcinoma Grade II
20	Hematuria	No specimen	Positive	Positive	Tissue fragment in cell block	Tumor visualized	Papillary transitional cell carcinoma, Grade I

TABLE III *Bladder Irrigation Cytology in 12 Cases* [illegible] *Cytologic Findings* [illegible] *Performed at the Los Angeles County—University of Southern California Medical Center*

Case No.	Reason for Cystoscopic Examination (Clinical history)	Results of Cytologic Examination			Urologists' Impression at Time of Cystoscopy	Tissue Diagnosis	Follow Up
		Catheterized Specimen	Irrigation Specimen	Cell Block			
21	Bilateral renal and [illegible] calculi	Suggestive of squamous carcinoma	Negative	Suspicious for carcinoma	Bullous edema of trigone	Chronic cystitis Cystitis cystica	None
22	Ureteral calculi	Suggestive of malignancy	Negative	No specimen	Ureteral calculi with edematous rt orifice	None	Negative
23	Filling defect in left ureter	Dysplastic squamous epithelial cells	Negative	No specimen	Ureteral tumor or calculi	Squamous metaplasia of bladder ureteral [illegible]	Dysplastic squamous epithelial cells in catheterized specimens
24	Cervical cancer	Negative	Atypical cells	No specimen	Bladder calculi removed	Cervical biopsy recurrent squamous ca	Negative. Patient had been irradiated
25	Cervical cancer	Negative	Abnormal cells	No specimen	Few areas of hemorrhagic mucosa	None	Negative. Patient had been irradiated
26	Treated bladder tumor "follow up" examination	Cells too degenerated for evaluation	Severe irradiation response	No specimen	Irradiation changes of mucosa. No tumor seen	None	Negative. Patient had been irradiated
27	Treated bladder tumor "follow-up" examination	Atypical cells	Atypical cells	No specimen	Possible recurrence of tumor	Chronic cystitis [illegible] ulcerations	Negative
28	Treated bladder tumor "follow up" examination	Atypical cells	Atypical cells	Negative	Possible recurrence of tumor	Severe chronic cystitis	Negative
29	Benign prostatic hyperplasia	Abnormal cells	Abnormal cells	No specimen	Chronic cystitis	Chronic cystitis Cystitis Cystica	Negative
30	Benign prostatic hyperplasia	Abnormal cells	Abnormal cells	No specimen	Cystitis Prostatic hyperplasia	None	Negative
31	Nephrocalcinosis	Mostly columnar cells	Cells greatly enlarged	No specimen	Normal bladder	None	None
32	Automobile accident	Cells too poorly preserved for evaluation		No specimen	Perforated bladder	None	None

Bladder specimens obtained with distilled water in the cytologic smears and in the cell blocks. The cells were small, being only slightly larger than lymphocytes, and appeared singly and in loose clusters. The anaplastic tumor cells were somewhat reminiscent of small cell carcinoma of the lung

Cases 7 and 8 In the first of these cases two small sessile tumors were seen by the urologist at the time of cystoscopy The biopsy taken at that time showed only chronic inflammation. In the second case a tumor was also visualized at cystoscopy and the biopsy was negative for malignancy Cytologic studies in both instances were diagnostic of transitional cell carcinoma. Because of the cytology reports of carcinoma, repeat biopsies were performed on both patients and confirmed the cytologic diagnoses.

Inconclusive or Atypical Cytologic Findings (Table III)

Of the 12 cases in which cytologic findings were inconclusive or indicated atypia, only nine were directly concerned with cells found in the irrigation specimen. In the other three cases the abnormal cells were found in the catheterized urine from patients with renal disease.

In these cases, three of the patients had been irradiated and four had chronic cystitis. In one case cellular distortion due to collection of the irrigation in distilled water made a diagnosis impossible. In another case, greatly enlarged but obviously benign cells were suggestive of a folic acid deficiency

Subsequent specimens and follow up studies of these patients was indicative of probable non-malignant disease

CYTOLOGIC DESCRIPTION OF THE URINARY BLADDER IRRIGATION SPECIMEN

The cellular samples obtained from the bladder irrigation specimen technique are distinctly different from those of either voided or catheterized urine and are, therefore, profusely illustrated.

Carcinoma

Bizarre nuclear configurations, coarse uneven chromatin clumping within irregular nuclear membranes, hyperchromasia, macronucleoli, and syncytial groups with great variation in size and shape, characterized the transitional cell carcinoma Grade II or greater (Plate I A–E) Nuclear/cytoplasmic ratios were altered in favor of the nucleus, except in the case of low grade carcinomas. In the irrigation specimens, cell populations were more than adequate for a definitive diagnosis.

Cells derived from transitional papilloma of the bladder or from Grade I papillary transitional cell carcinoma were not identified as being malignant (Plate I F) In some instances cells from these lesions were recognized as being atypical (Plate I G) but a definitive diagnosis could be made in these cases only if fragments of carcinoma appeared in the cell block preparations (Plate I H and I)

In evaluating voided urine, expanded criteria of malignancy (i.e., the presence of benign, non-ciliated columnar cells, elongated transitional cells, palisade formations, benign papillary fronds, or *increased cellularity) have been used to improve* the detection of these low grade tumors by some authors.[1, 8, 10, 14, 21]

All of these criteria were found frequently in the "normal" bladder irrigation specimen and could not be considered as suspicious for malignancy in these specimens.

Normal Epithelium

The predominate, non-malignant cells were the well-differentiated columnar and the transitional epithelium (Plate II A) Variation in morphology was common, even within the same specimen.

Transitional Cells Transitional cells exhibited great variation in shape and size. They appeared singly and in sheets. The single cell ranged in size *from 10 to 150 µ and contained from one to 55* nuclei. It was polyhedral or elongated in shape Sheets varied from pavements of polyhedral cells with abundant cytoplasm (Plate II B) to plaques composed of small round cells with scant cytoplasm (Plate II C) In sheets, the individual cells usually contained no more than two nuclei. The transitional cell was recognized by its nuclear characteristics. The pale staining nucleus was encircled by a dense membrane. A few fine or medium-sized chromatin clumps were scattered *throughout or arranged peripherally in an other*wise empty-appearing structure. Single or multiple, round or irregularly-shaped eosinophilic nucleoli were usually present (Plate II D)

Cytoplasm was dense, opaque and usually cyanophilic. In some cases brush-like borders extended from the cytoplasmic membrane (Plate II E)

Multiple vacuoles were almost always present (Plate II F) They differed from vacuoles of secretion and regeneration by their "dry" appearance, resembling wrinkles or blisters possibly caused by stretching or uneven thinning of the cytoplasmic membrane.

Transitional cells in the bladder irrigation did not differ appreciably in appearance from those described in voided or catheterized urine. The only possible exception was the multinucleated (or giant multinucleated) transitional cell reported to be characteristic of catheterized urine of ureteral or renal pelvic origin,[14, 8] and which has been found in the tubules of the human kidney [8] Such cells appeared frequently in bladder

TABLE I. *Bladder Irrigation Studies on 12 [illegible] in Which Cytologic Findings [illegible] at the [illegible] Angeles County—University of Southern California Medical Center*

Case No.	Reason for Cytological Examination (Clinical History)	Results of Cytologic Examination			Cystoscopic Impression at Time of Cystoscopy	Tissue Diagnosis	Follow Up
		Catheterized Specimen	Irrigation Specimen	Cell Block			
21	Bilateral renal staghorn calculi	Suggestive of squamous carcinoma	Negative	Suspicious for carcinoma	Bullous edema of trigone	Chronic cystitis. Cystitis cystica	None
22	Ureteral calculi	Suggestive of malignancy	Negative	No specimen	Ureteral calculi with edematous ureteral orifice	None	Negative
23	Filling defect—left ureter	Dysplastic squamous epithelial cells	Negative	No specimen	Ureteral tumor or calculi	Squamous metaplasia of bladder ureteral calculi	Dysplastic squamous epithelial cells in catheterized specimens
4	Cervical cancer	Negative	Atypical cells	No specimen	Bladder calculi removed	Cervical biopsy recurrent squamous ca	Negative. Patient had been irradiated
25	Cervical cancer	Negative	Abnormal cells	No specimen	Few areas of hemorrhagic mucosa	None	Negative. Patient had been irradiated
26	Treated bladder tumor "follow-up" examination	Cells too degenerated for evaluation	Severe radiation response	No specimen	Irradiation changes of mucosa. No tumor seen	None	Negative. Patient had been irradiated
27	Treated bladder tumor "follow-up" examination	Atypical cells	Atypical cells	No specimen	Possible recurrence of tumor	Chronic cystitis with ulcerations	Negative
28	Treated bladder tumor "follow-up" examination	Atypical cells	Atypical cells	Negative	Possible recurrence of tumor	Severe chronic cystitis	Negative
29	Benign prostatic hyperplasia	Abnormal cells	Abnormal cells	No specimen	Chronic cystitis	Chronic cystitis. Cystitis Cystica	Negative
30	Benign prostatic hyperplasia	Abnormal cells	Abnormal cells	No specimen	Cystitis. Prostatic hyperplasia	None	Negative
31	Nephrocalcinosis	Mostly columnar cells	Cells greatly enlarged	No specimen	Normal bladder	None	None
32	Automobile accident	Cells too poorly preserved for evaluation		No specimen	Perforated bladder	None	None

Bladder specimens obtained with distilled water in the cytologic smears and in the cell blocks. The cells were small, being only slightly larger than lymphocytes, and appeared singly and in loose clusters. The anaplastic tumor cells were somewhat reminiscent of small cell carcinoma of the lung

Cases 7 and 8. In the first of these cases two small sessile tumors were seen by the urologist at the time of cystoscopy The biopsy taken at that time showed only chronic inflammation. In the second case a tumor was also visualized at cystoscopy and the biopsy was negative for malignancy Cytologic studies in both instances were diagnostic of transitional cell carcinoma. Because of the cytology reports of carcinoma, repeat biopsies were performed on both patients and confirmed the cytologic diagnoses.

Inconclusive or Atypical Cytologic Findings (Table III)

Of the 12 cases in which cytologic findings were inconclusive or indicated atypia, only nine were directly concerned with cells found in the irrigation specimen. In the other three cases the abnormal cells were found in the catheterized urine from patients with renal disease.

In these cases, three of the patients had been irradiated and four had chronic cystitis In one case cellular distortion due to collection of the irrigation in distilled water made a diagnosis impossible. In another case, greatly enlarged but obviously benign cells were suggestive of a folic acid deficiency

Subsequent specimens and follow up studies of these patients was indicative of probable non-malignant disease.

CYTOLOGIC DESCRIPTION OF THE URINARY BLADDER IRRIGATION SPECIMEN

The cellular samples obtained from the bladder irrigation specimen technique are distinctly different from those of either voided or catheterized urine and are, therefore, profusely illustrated.

Carcinoma

Bizarre nuclear configurations, coarse uneven chromatin clumping within irregular nuclear membranes, hyperchromasia, macronucleoli, and syncytial groups with great variation in size and shape, characterized the transitional cell carcinoma Grade II or greater (Plate I A–E) Nuclear/cytoplasmic ratios were altered in favor of the nucleus, except in the case of low grade carcinomas. In the irrigation specimens, cell populations were more than adequate for a definitive diagnosis.

Cells derived from transitional papilloma of the bladder or from Grade I papillary transitional cell carcinoma were not identified as being malignant (Plate I F) In some instances cells from these lesions were recognized as being atypical (Plate I G) but a definitive diagnosis could be made in these cases only if fragments of carcinoma appeared in the cell block preparations (Plate I H and I)

In evaluating voided urine, expanded criteria of malignancy (i.e., the presence of benign, non-ciliated columnar cells, elongated transitional cells, palisade formations, benign papillary fronds, or increased cellularity) have been used to improve the detection of these low grade tumors by some authors.[1] [8] [16] [21] [29]

All of these criteria were found frequently in the "normal" bladder irrigation specimen and could not be considered as suspicious for malignancy in these specimens.

"Normal" Epithelium

The predominate, non-malignant cells were the well-differentiated columnar and the transitional epithelium (Plate II A) Variation in morphology was common, even within the same specimen.

Transitional Cells Transitional cells exhibited great variation in shape and size. They appeared singly and in sheets. The single cell ranged in size from 10 to 150 μ and contained from one to 55 nuclei. It was polyhedral or elongated in shape Sheets varied from pavements of polyhedral cells with abundant cytoplasm (Plate II B) to plaques composed of small round cells with scant cytoplasm (Plate II C) In sheets, the individual cells usually contained no more than two nuclei. The transitional cell was recognized by its nuclear characteristics. The pale staining nucleus was encircled by a dense membrane. A few fine or medium-sized chromatin clumps were scattered throughout or arranged peripherally in an otherwise empty appearing structure. Single or multiple, round or irregularly-shaped eosinophilic nucleoli were usually present (Plate II D)

Cytoplasm was dense, opaque and usually cyanophilic. In some cases brush-like borders extended from the cytoplasmic membrane (Plate II E)

Multiple vacuoles were almost always present (Plate II F) They differed from vacuoles of secretion and regeneration by their "dry" appearance, resembling wrinkles or blisters possibly caused by stretching or uneven thinning of the cytoplasmic membrane.

Transitional cells in the bladder irrigation did not differ appreciably in appearance from those described in voided or catheterized urine. The only possible exception was the multinucleated (or giant multinucleated) transitional cell reported to be characteristic of catheterized urine of ureteral or renal pelvic origin,[14] [8] and which has been found in the tubules of the human kidney [9] Such cells appeared frequently in bladder

TABLE 1. Bladder Irrigation Cytology in 12 Cases in Which Cytologic Findings [illegible] Interpreted at the [illegible] County—University of Southern California Medical Center

Case No.	Reason for Cytologic Examination (Clinical History)	Results of Cytologic Examination			Cystoscopic Impression at Time of Cystoscopy	Tissue Diagnosis	Follow Up
		Catheterized Specimen	Irrigation Specimen	Cell Block			
1	Bilateral renal staghorn calculi	Suggestive of squamous carcinoma	Negative	Suspicious for carcinoma	Bullous edema of mucosa	Chronic cystitis. Cystitis cystica	None
22	Ureteral calculi	Suggestive of malignancy	Negative	No specimen	Ureteral calculi with edematous mucosa	None	Negative
23	Filling defect in left ureter	Dysplastic squamous epithelial cells	Negative	No specimen	Ureteral tumor or calculi	Squamous metaplasia of bladder; ureteral calculi	Dysplastic squamous epithelial cells in abnormal specimens
24	Cervical cancer	Negative	Atypical cells	No specimen	Bladder calculi removed	Cervical biopsy, recurrent squamous ca	Negative. Patient had been irradiated
25	Cervical cancer	Negative	Abnormal cells	No specimen	Few areas of hemorrhagic mucosa	None	Negative. Patient had been irradiated
26	Treated bladder tumor "follow-up" examination	Cells too degenerated for evaluation	Severe irradiation reaction	No specimen	Irradiation changes of mucosa. No tumor seen	None	Negative. Patient had been irradiated
27	Treated bladder tumor "follow-up" examination	Atypical cells	Atypical cells	No specimen	Possible recurrence of tumor	Chronic cystitis, residual carcinoma	Negative
28	Treated bladder tumor "follow-up" examination	Atypical cells	Atypical cells	Negative	Possible recurrence of tumor	Severe chronic cystitis	Negative
29	Benign prostatic hyperplasia	Abnormal cells	Abnormal cells	No specimen	Chronic cystitis	Chronic cystitis. Cystitis cystica	Negative
30	Benign prostatic hyperplasia	Abnormal cells	Abnormal cells	No specimen	Cystitis. Prostatic hyperplasia	None	Negative
31	Nephrocalcinosis	Mostly columnar cells	Cells greatly enlarged	No specimen	Normal bladder	None	None
32	Automobile accident	Cells too poorly preserved for evaluation		No specimen	Perforated bladder	None	None

Bladder specimens obtained [illegible] b distilled water

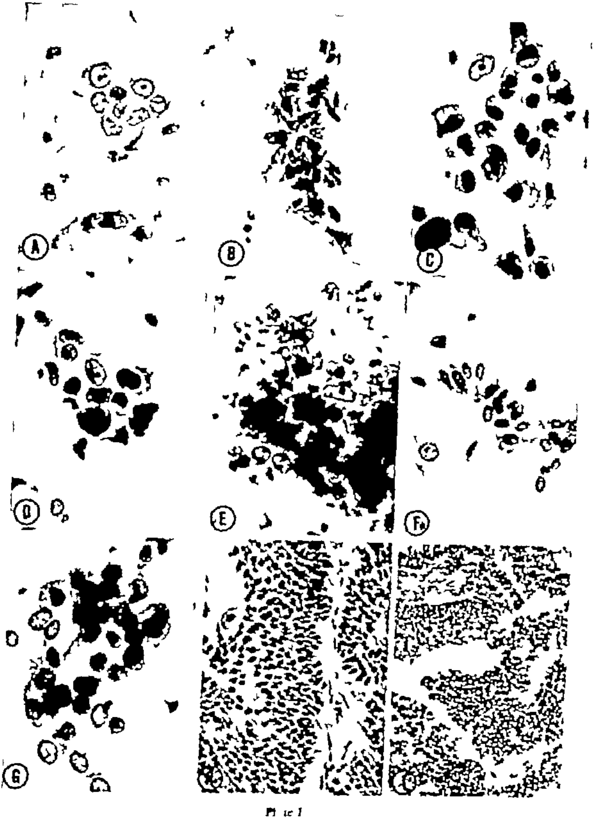

Plate I

Bladder irrigation specimens. A–E) Carcinoma cells: note high nuclear/cytoplasmic ratio, variation in nuclear size and staining reaction, and chromatin clumping. F) Benign columnar and transitional epithelial cells (Case 1, Table II); compare with Plate II A); diagnosis based on tissue fragment in cell block (H). G) Sheet of atypical transitional epithelial cells (Case 2, Table II); cells are not considered sufficiently abnormal to permit a definitive diagnosis of carcinoma. Diagnosis based on tissue fragment in cell block (see I). H) Tissue fragment in cell block prepared from bladder irrigation centrifugate (Case 1, Table II) showing transitional cell carcinoma, grade I. Cells identifiable as carcinoma were not found in smears or membrane filter preparations (see F). I) Tissue fragment in cell block prepared from irrigation centrifugate (Case 2, Table II) showing papillary transitional cell carcinoma, grade II. No cells identifiable as carcinoma in smears of membrane filter preparations (see G). (A–G × 500; H × 230; I × 150)

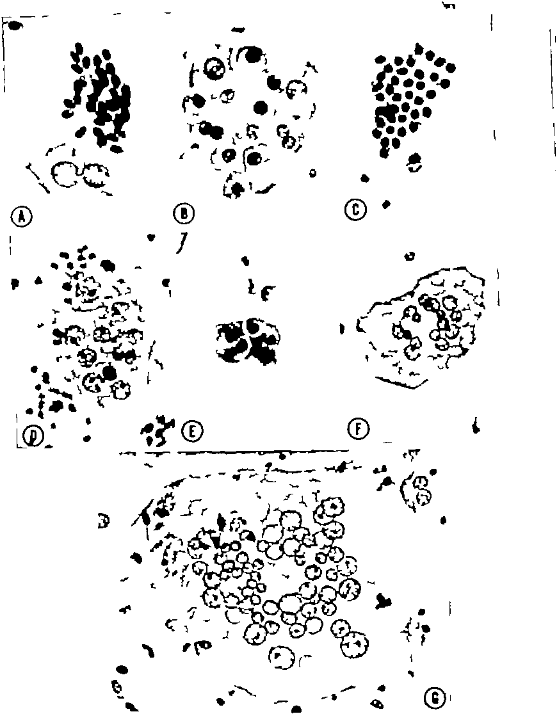

Plate II

Bladder irrigation specimens. A) Columnar and transitional epithelial cells. B and C) Sheets of transitional cells. D) Multinucleated transitional epithelial cell with prominent acidophilic nucleoli. E) Groups of transitional cells with peculiar cuticle. F) Multinucleated transitional cell with cytoplasmic vacuolization. G) Giant, multinucleated transitional cell. Compare in size with transitional cell in upper right hand corner and columnar cells in background (× 500)

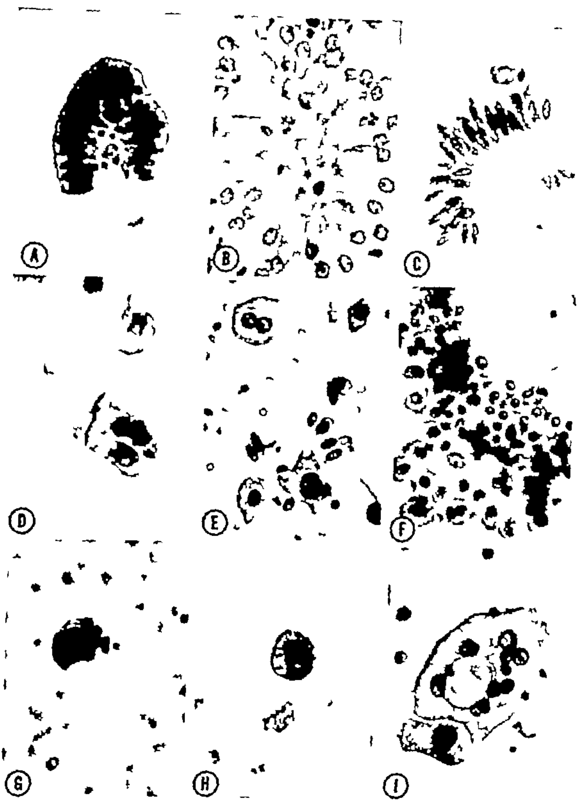

Plate III

Bladder irrigation specimens A) Columnar cells with papillary configuration, "papillary frond" B) Columnar cells, rosette effect. C) Columnar cells, apparently oriented toward a surface and possibly exhibiting terminal bars. D) Ciliated columnar epithelial cells. E) Transitional and columnar epithelial cells typical of the bladder irrigation specimen. *The frequent occurrence of columnar cells strongly suggests that at least some of these columnar cell forms exfoliate from the bladder epithelium.* F) Large sheet of transitional epithelial cells. Note mitotic figure. G) Multinucleated histiocyte Note foamy cytoplasm. Compare with multinucleated transitional epithelial cells in Plate I G and I H) Acidophilic intracytoplasmic inclusions in histocyte. I) Multinucleated transitional epithelial cell with intracytoplasmic inclusion. These inclusions are relatively large and red-brown or yellow-brown in color (A–C, × 500 D × 1000 E–I × 400) irrigations (Plate II G) Usually they were few in number but in some instances they were the predominant cell type. In this study 18 retrograde catheterization specimens were received from the renal pelves. Multinucleated transitional cells appeared in only half of these and none of the individual cells equaled either in size or in the number of nuclei those found in the bladder irrigation.

Columnar Cells Columnar cells appeared singly in sheets, papillary fronds, rosette formations, or in honeycomb and/or grape-like clusters (Plate III A-C) Nuclear chromatin was finely granular and evenly dispersed within definite border Nucleoli were rare Hyperchromasia was common Staining reaction of the translucent cytoplasm was either intermediate or cyanophilic Terminal plates were frequently present and at times cilia were clearly visible (Plate III D)

It is known that, occasionally areas of the bladder are lined by single layer of columnar mucus-producing cells,[13] and that portion of the male urethra is lined with stratified or pseudostratified columnar epithelium[20] The epithelium lining the tubules and ducts of the prostate can give rise to non-ciliated cuboidal and columnar cells[20] Also, in both sexes similar cells can be shed from the epithelium lining the lacunae of Morgagni and the glands of Littre.

Non-ciliated columnar cells appeared in the majority of bladder irrigations. Ciliated columnar cells appeared less frequently The clinical histories of the patients with columnar cells in their irrigation samples were so varied that neither the origin of these cells nor their correlation to specific disease entity was possible However the frequent occurrence of columnar cells in bladder irrigations strongly suggests that at least some of them must be derived from the lining of the urinary bladder

Transitional and columnar cells, co-existing within the same specimen, characterized the bladder irrigation specimen. Usually they were found in more than one of their morphologic forms. Thus typical irrigation might contain single transitional cells of various sizes, few pavement cells and giant multinucleated cells along with single and clustered columnar cells (Plate III E) Small numbers of intermediate and superficial squamous cells were found in many specimens also. Mitotic figures were frequently present (Plate III F)

Non-Epithelial Elements. Polymorphonuclear leukocytes, red blood cells, bacteria, fungi, trichomonads, spermatozoa, casts, microcalculi, and drug crystals were observed occasionally Single and multinucleated histiocytes were identified by their foamy cytoplasm and characteristic nuclei (Plate III G)

Intracytoplasmic eosinophilic inclusion bodies as described by Melamed and Wolinska[21] were found in over one-half of the irrigation specimens (Plate III H) Generally the cell containing the inclusion resembled histiocyte with degenerated nucleus, but in three cases the cell appeared to be transitional in origin Other cytoplasmic inclusions appeared frequently in well-preserved *transitional cells, especially in their giant multinucleated form* (Plate III I)

Intranuclear inclusions were found in three of our cases Viral inclusions were found in voided urine collected from female patient during follow up studies for suspected renal disease (Plate IV A) Eosinophilic intranuclear inclusions were observed in the irrigation specimens of two other cases (Plate IV B) One of these patients had adenocarcinoma of the colon and the other had adenocarcinoma of the urethra.

Calcified concretions with peripheral concentric laminations were found in 15 specimens (Plate IV C) All 15 patients were males and tissue sections revealed that 12 had benign prostatic hyperplasia and three had adenocarcinoma of the prostate It was assumed, therefore, that these concretions were corpora amylacea from the prostate gland.

Post Irradiation Effect Cellular alterations suggesting response to irradiation in bladder irrigations were similar to those described in urine specimens [14] Irradiation reactions of epithelial cells presented in several ways In some *cases there was predominance of binucleated* or multinucleated forms (Plate IV D) In others, extreme enlargement of cells was noted with proportionate enlargement of nuclei (Plate IV E) Often large vacuoles filled the entire cell causing the nuclei to protrude peripherally (Plate IV F) Although cells with these characteristics were found in normal" irrigations, they were few in number and scattered throughout the smear In the presence of irradiation the altered cell predominated or were very numerous.

Squamous Metaplasia A cytologic diagnosis of squamous metaplasia of the bladder was suggested in the irrigations of both males and females by predominance of flat polyhedral cells with pyknotic or vesicular nuclei Eosinophilia and cytoplasmic granulation were frequently observed in these specimens But since estrogen therapy various drugs and the normal cycle of the premenopausal female can elicit the same response from bladder epithelium,[28] a definitive diagnosis of metaplasia was never attempted.

Atypical Epithelium. In the nine irrigations where definitive diagnosis was not made, the abnormal cells were found in isolated sheets (Plate IV G-I) scattered throughout the smear and were less

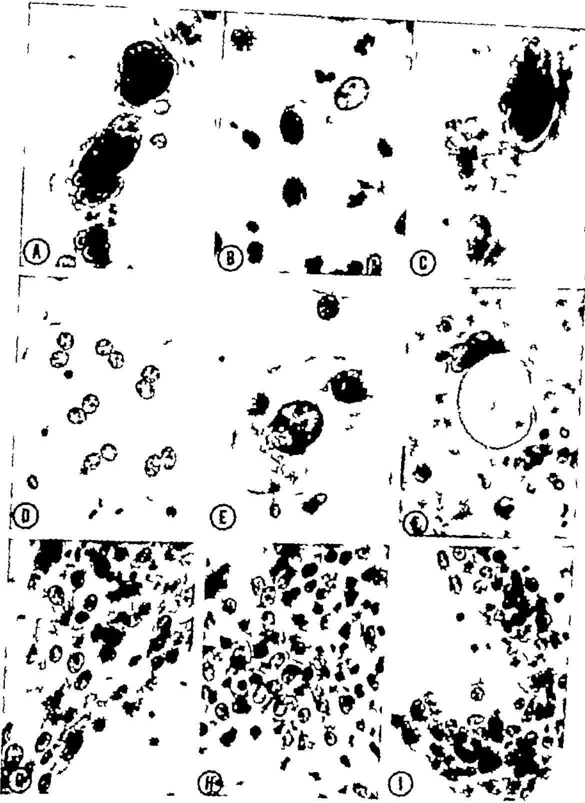

Plate IX

A) Voided urine. Intranuclear viral inclusions (× 500) B) Bladder irrigation specimen. Intranuclear inclusions. These brightly acidophilic inclusions were found in two patients with adenocarcinoma (one had adenocarcinoma of the colon, one had adenocarcinoma of the urethra) (× 1500) C) Bladder irrigation specimen. Corpora amylacea, assumed to be of prostatic origin (× 500) D) Irradiation reaction, transitional epithelium. Note frequency of binucleation (× 500) E) Bladder irrigation specimen. Irradiation reaction, transitional epithelium. Note increased nuclear and cell size (× 500) F) Bladder irrigation specimen. Irradiation reaction showing transitional epithelial cells with extreme vacuolization of cytoplasm (× 500) G) Bladder irrigation specimen. Sheet of typical transitional cells from patient with chronic cystitis (× 500) H) Bladder irrigation specimen. Sheet of atypical cells from patient irradiated for cervical carcinoma (× 500) I) Bladder irrigation specimen. Sheet of atypical transitional cells from patient with chronic cystitis (× 500) numerous than in preparations from patients with malignancy

Follow-up studies failed to reveal carcinoma in any of these nine cases.

DISCUSSION

Urinary cytology has not received widespread acceptance by the urologist, probably because an acceptable degree of diagnostic accuracy is usually associated with high incidence of false-positive results As pointed out in the introduction, wide statistical variation in accuracy must indicate an inherent defect in the method.

In previous studies either voided or catheterized urine was used. One of the problems encountered most frequently in the microscopic evaluation of these specimens include scant cellular yield and poor preservation of cellular morphology

Prior attempts to improve the cellular yield and preservation of cells have included concentration techniques, having the patient exercise prior to voiding,[11] collection of repeat specimens, and immediate fixation of specimens These techniques neither affect the tumor which does not readily exfoliate cells nor aid in the preservation of cells which have already undergone degeneration during their period of storage in the urinary bladder

Bladder irrigation specimens consistently yielded adequate, well preserved cellular material for microscopic evaluation, thus overcoming this problem

Another difficulty encountered in urine cytology is the inability of the cytopathologist to distinguish between cells derived from well-differentiated papillary transitional cell carcinoma and those derived from normal transitional epithelium or from epithelium affected by inflammatory or other non-malignant conditions. In fact, it has been stated that low grade papillary transitional cell carcinoma and papilloma of the bladder are neoplasms which cannot be recognized on the basis of cellular criteria[4] alone and will be diagnosed only if tissue fragment appears in the smeared sediment or the cell block.

Such tissue fragments were found in half of the cell blocks prepared from the bladder irrigations of patients with malignancy In one case the tissue fragment was diagnostic of low grade tumor not visualized cystoscopically In another case the low grade tumor was seen cystoscopy but was not confirmed on first biopsy

Some authors have tried to improve the detection of well differentiated papillary transitional cell carcinomas by broadening the criteria of malignancy to include additional architectural and cellular parameters. In voided urine specimens, these authors have considered the following as suspicious for neoplasm presence of benign, nonciliated columnar cells, elongated transitional cells, palisade formations, benign papillary fronds and even increased cellularity Because all these criteria have been described in the absence of papillary carcinoma of the bladder their appearance in urine sediments is of limited diagnostic value and will only serve to increase the number of false-positive cytology reports.

Crabbe pioneered the use of these expanded criteria when screening workers in the dyestuffs industry of England. These workers had been exposed to known bladder carcinogens Crabbe was restricted to the examination of voided urine sediments and cystoscopic examination of the bladder was not carried out unless his cytologic report indicated an abnormality Under these circumstances he realized that false-positive results were preferred to false-negative results if early detection and proper control of occupational tumors was to be accomplished He also knew that false-positive reports based on these expanded criteria would not be acceptable in routine cytologic practice. Others who have subsequently adopted his criteria may not have been aware of his indications for their use.

Since these cellular criteria, used by Crabbe and others in evaluating voided urine, are present in the majority of normal bladder irrigations, they cannot be applied as diagnostic of malignancy in these specimens For these reasons the criteria used in evaluating bladder irrigation samples are not expanded or contrived, but employ principles similar to those used in evaluating cells from other organ systems.

There were no false-positive diagnoses in this study There were only nine cases in which cellular material was considered atypical or inconclusive for a definitive diagnosis.

In spite of the cited problems associated with currently employed techniques, the value of urinary cytology has been established in the diagnosis of lesions arising from the transitional epithelium (urothelium) of the urinary tract including urethra, urinary bladder ureters, and renal pelvis Urinary cytology has proven to be particularly helpful in the detection of in situ and early invasive, nonpapillary carcinoma of the bladder because cystoscopic findings in these patients may be normal or indistinguishable from those of inflammatory or other benign conditions

Periodic cytologic examination of urine is indispensable for the earliest detection of recurrent carcinoma in patients with previously treated bladder tumors, and in the earliest detection of carcinoma developing in industrial workers

Vol 15 No 4

who have been exposed to known bladder carcinogens.[2 8 18 19]

Also there have been numerous reports of the cytologic detection of cancer in patients where cystoscopic examination failed to reveal the lesion.[8 12 17 1 18] It is also recognized that cystoscopic biopsy of suspicious areas is not always feasible when the examination is being performed under anesthesia and that there are areas in the urinary bladder which are difficult to visualize or biopsy.

Because patients undergoing cystoscopy are a highly suspect group, the urologist should consider utilizing any method which could provide him with primary diagnostic information. Cytologic evaluation of bladder irrigation specimens is one such method. As an adjunct to cystoscopy the technique is simple, consumes little time, requires no special equipment and is relatively painless to the patient.

In this study there were three cases in which carcinoma not seen cystoscopically was detected by cytology. In three other cases, carcinoma seen cystoscopically was confirmed as malignant by cytology but not by the first biopsy.

As an adjunct to cystoscopy the cytologic evaluation of bladder irrigations can provide the urologist with valuable information which will enhance the sensitivity of this diagnostic procedure.

SUMMARY

Three hundred thirty five bladder irrigations collected from patients undergoing cystoscopy examination in the Urology Services of Los Angeles County—University of Southern California Medical Center are the subject of this report.

In 20 cases of proven carcinoma, findings in cytology, cystoscopy and histopathology are correlated. Twelve cases are described in which cytologic interpretation was inconclusive or indicated atypia.

Based upon microscopic evaluation of cellular morphology and cellular spread, the cytology of "normal" and malignant bladder irrigation specimens is described in detail. The cellular presentation of bladder irrigation is compared with that of catheterized urine from the same patient.

The value of the bladder irrigation as an adjunct to cystoscopy is discussed.

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Cytologic Differentiation of Carcinoma of the Breast by Electron Microscopy

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Various classifications of carcinoma of the breast have been proposed.[1,2,3,4,5] These classifications, although useful in a series of cases, are of little help in the evaluation of a single case. The failure of existing light microscopic classifications to predict the prognosis and response to pharmacologic agents is mainly due to the variation in histologic pattern of breast carcinoma. This variation frequently is seen in different parts of the same tumor and also in the metastases. Therefore another method for classifying breast cancer is needed.

Previously we proposed that medullary carcinoma originates from ductal epithelial cells and the scirrhous variety from myoepithelial cells.[8] In our study on fibroadenomas and normal breast we showed that myoepithelial cells carry on their plasma membrane certain enzymes that are lacking in ductal and ductular epithelial cells.[9] In the present study breast carcinomas were examined by light microscopy, electron microscopy, and light and electron-microscopic histochemistry. The study revealed that breast carcinoma may arise from myoepithelial cells, ductal epithelial cells, or ductular epithelial cells of the secretory unit of the breast. It is suggested that the behavior of the tumor is related to its cellular origin.

MATERIAL AND METHODS

Breast carcinomas were collected from surgical material selected at random. The only information used at the time of collecting the tissue was the name, date, diagnosis (limited to carcinoma or benign) and the surgical pathology number. The cases were then classified in my laboratory according to the enzyme reaction and electron microscopic morphology into myoepithelial, ductal epithelial and ductular epithelial carcinomas. The surgical pathology slides were reviewed afterward and the results were compared.

Biopsies were fixed in glutaraldehyde[11] for one hour and then washed in phosphate buffer. Frozen sections 40 μ thick were cut, placed in cold 0.3 M sucrose and incubated in the various enzyme substrates for 10 to 30 minutes. The enzymes tested for were adenosine triphosphatase (ATPase), inosine diphosphatase (IDPase),[13] alkaline phosphatase (Alkpase)[4] and acid phosphatase (Acpase). After incubation a few sections were treated with 1% ammonium sulfide for two minutes, washed and mounted on slides, and studied with a light microscope. The remaining sections were washed in 0.3 M sucrose, postfixed in 1% OsO_4[13] in phosphate buffer for one hour, and processed for electron microscopic study. Tissue was embedded in DER.[7] Thin sections were cut and stained with uranyl acetate[14] for enzyme localization only and with uranyl acetate and lead citrate[11] for ultrastructural classification of the tumor. Hematoxylin and eosin stained sections were reviewed and the findings were compared with those observed by electron microscopy and histochemistry.

RESULTS

The carcinomas of the breast were classified as myoepithelial, ductal epithelial or ductular epithelial, or ductular epithelial according to their cells of origin in the secretory unit of the breast.

Myoepithelial carcinoma

Myoepithelial carcinoma arises from the myoepithelial cells surrounding the ducts and ductules of the secretory unit of the breast. Twelve cases were studied; one showed axillary lymphnode metastasis. The average age of the patients was 54 years. With light microscopy, the myoepithelial cell carcinoma showed strands of neoplastic cells separated from a dense collagenizing stroma by a narrow space (Fig. 1). The neoplastic cells and the collagenous stroma, although intimately associated, appeared as two distinct types of tissue. This form of tumor cell infiltration was not the one most frequently observed but was the one most easily recognized. Other forms included the formation of sheets of tumor cells, intraductal carcinoma, and the infiltration by small groups of tumor cells. These patterns were seen in tumors from different patients or in different parts of the same tumor. With the electron microscope, the myoepithelial cell carcinomas showed large nuclei with a dense chromatin pattern and areas of hetero chromatin at the nuclear border. The cytoplasm consisted of poorly developed agranular

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Presented at the 17th Annual Meeting of the American Society of Cytology, Chicago, 1969.

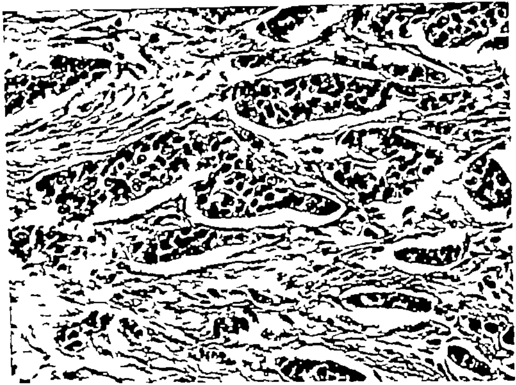

Fig 1 Myoepithelial carcinoma showing group of tumor cells in dense hyalinized and collagenized stroma. The neoplastic cells are separated from the surrounding stroma by narrow spaces. (×250)

endoplasmic reticulum with free ribosomes (Fig. 2) Mitochondria were few and small with distorted cristae and frequently were seen grouped in different parts of the cell. Filaments, normal feature of myoepithelial cells, often were lacking in malignant myoepithelial cells and when present frequently were distorted (Fig 3) As did the normal myoepithelial cells, the malignant myoepithelial cells gave positive reaction for ATPase and IDPase on the plasma membrane (Figs 2 and 3) Acid phosphatase when positive was limited to the lysosomes, which, however are rarely encountered in malignant myoepithelial cells. Alkaline phosphatase, an enzyme present in normal myoepithelial cells, was absent in the malignant myoepithelial cells but was found in the capillaries of the tumor (Fig. 4).

Ductal epithelial carcinoma

Eleven cases of ductal epithelial carcinoma were studied and one showed lymphnode metastases. Most of the patients were over 50 years of age and the average age was 60 years With the light microscope, the ductal epithelial cancers formed sheets of tumor cells separated by a small amount of collagen (Fig 5) The tumor cells were large and polyhedral with frequent small vacuoles in their cytoplasm. These vacuoles were seen either within the cytoplasm of the malignant cells or between adjacent neoplastic cells. The lymphocytic infiltration seen in some ductal tumors was not consistent finding and could also be seen in other types of breast carcinoma.

With the electron microscope, the ductal carcinoma was characterized by large polyhedral cells with large indented nuclei and prominent nucleoli. The nucleolus had prominent nucleolonema and small pars amorpha. The cytoplasm was large and contained many ribosomes and polysomes with occasional strands of rough-surfaced endoplasmic reticulum. Intracytoplasmic and intercellular vacuoles, either lined by short microvilli or smooth membrane, were consistently present (Fig 6) These lumen-like structures corresponded to the small vacuoles seen with the light microscope. The enzymes ATPase IDPase, and alkaline phosphatase were absent in malignant ductal epithelial cells. Acid phosphatase was localized in the lysosomes (Fig 7)

Ductular epithelial carcinoma

Fourteen cases of ductular carcinoma were included in our study eight of which showed lymph

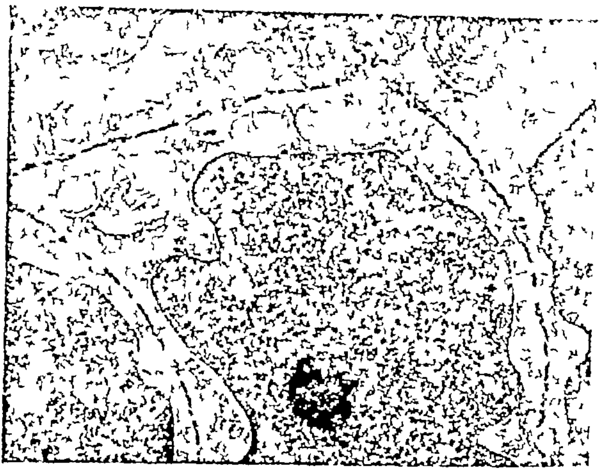

FIG. Myoepithelial cancer showing large nuclei with dense and homogeneously distributed chromatin. Notice the small Golgi, the agranular endoplasmic reticulum and the aggregate of mitochondria in one part of the cell. The tissue was incubated in medium containing ATP substrate and lead precipitate is seen localized to the plasma membrane of the neoplastic cells. (×15,500)

node metastasis. The average age of these patients was 50 years and four were under 40 years of age. Histologically, ductular carcinoma may show a lobular pattern, it may infiltrate a reticular type of stroma as single cells, or it may form small acini of tumor cells surrounded by a delicate stroma (Figs. 8 and 9). Because of the histologic similarity to the other two types of carcinoma of the breast, it is often not recognized by light microscopy. The cytoplasm of the tumor cells frequently appears clear, a feature which can be recognized also in the metastatic cells in the lymphnodes.

At the ultrastructural levels, several variations in the cytologic features of the ductular carcinoma were observed. The nucleus was ovoid in the *in-situ* form and was elongated with irregular infolding of the membrane in the invasive form. Patches of heterochromatin were scattered throughout the nucleoplasm, and the nucleolus showed a large pars amorpha and a small nucleolonema. The cytoplasm contained either a poorly developed agranular or a well-developed rough-surfaced endoplasmic reticulum. Glycogen particles and lipid droplets were sometimes found in the cytoplasm. Variation in the size, shape, and number of the mitochondria was a prominent feature. Most of the tumor cells had small mitochondria with very dense matrices and a few dilated cristae. The malignant ductular cells appeared less cohesive than those of ductal or myoepithelial origin (Fig. 10). The plasma membranes of adjacent tumor cells frequently were separated by spaces containing microvilli. Small lumens lined by short microvilli were sometimes encountered. The interdigitation of the microvilli was frequently so dense among adjacent tumor cells that no true lumen could be recognized. Enzyme reactions for ATPase, IDPase and alkaline phosphatase were negative. Acid phosphatase was present in the cytoplasmic ground substance and in the heterochromatin and the nucleolonema of the nuclei (Figs. 11 and 12). Lysosomes when present also gave a positive reaction for acid phosphatase. No reaction for acid phosphatase was observed in the mitochondria, lipid droplets, and Golgi apparatus.

Discussion

Since the behavior of any tumor depends mostly upon the cell of its origin, a classification of carcinoma of the breast according to this principle

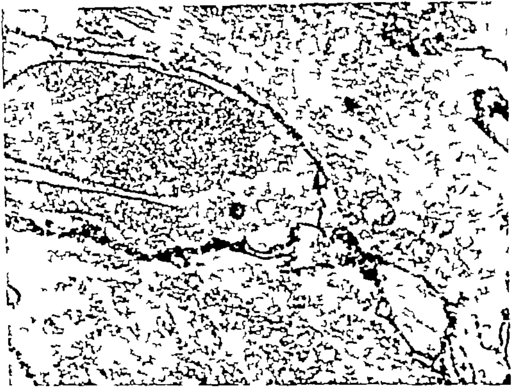

Fig. 3 Myoepithelial cancer with positive reaction for ATPase. Notice the fine filaments in the cytoplasm of some tumor cells (×15,500)

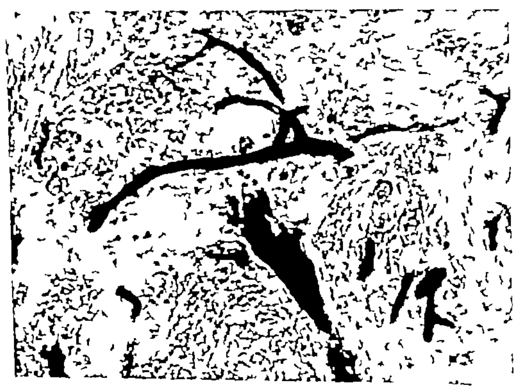

Fig. 4 Myoepithelial carcinoma showing positive reaction for alkaline phosphatase in the capillaries feeding the tumor. Alkaline phosphatase is absent from the neoplastic cells (×400)

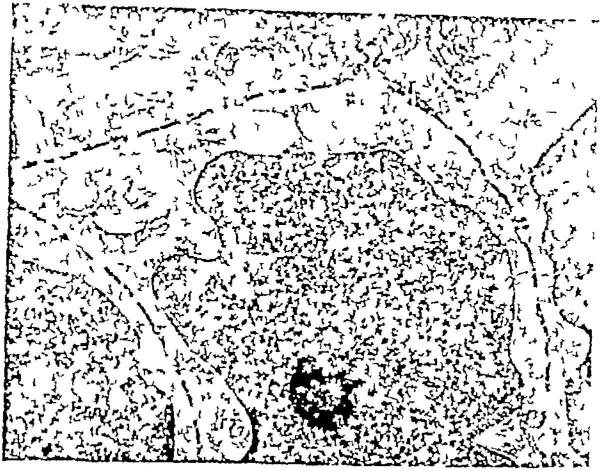

Fig. 2. Myoepithelial cancer showing large nuclei with a dense and homogeneously distributed chromatin. Notice the small Golgi, the agranular endoplasmic reticulum and the aggregate of mitochondria in one part of the cell. The tissue was incubated in a medium containing ATP substrate and lead precipitate is seen localized to the plasma membrane of the neoplastic cells. (×13,500)

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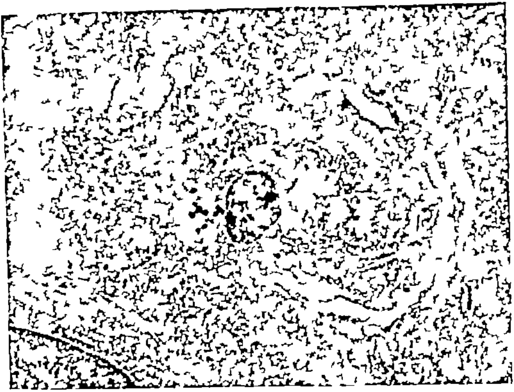


Fig 7 Ductal epithelial cancer showing positive reaction for acid phosphatase in the lysosomes. Notice the lack of the reaction in the Golgi apparatus. (×30,500)

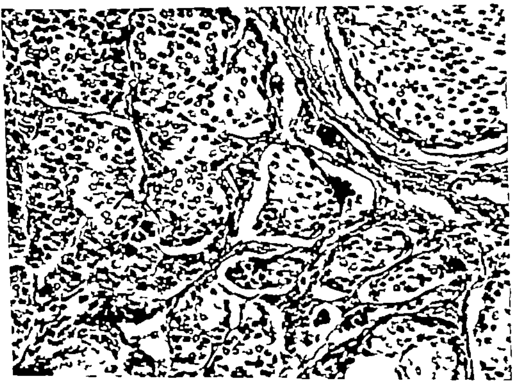


Fig 8 Infiltrating ductular carcinoma showing groups of tumor cells in nodular patterns separated by delicate stroma. Notice the pale staining cytoplasm of the neoplastic cells (×250)

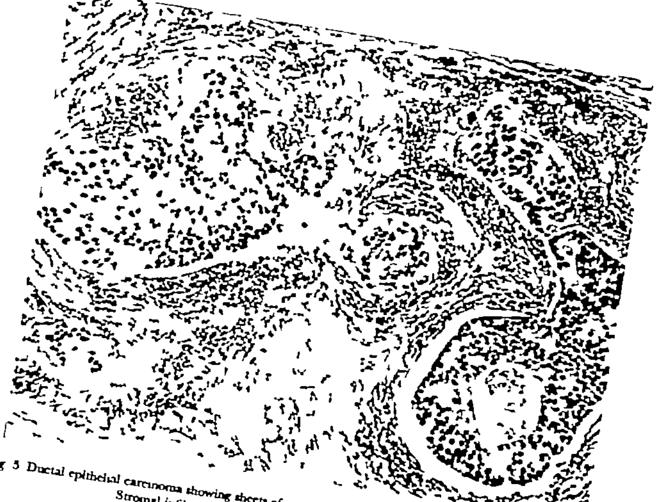
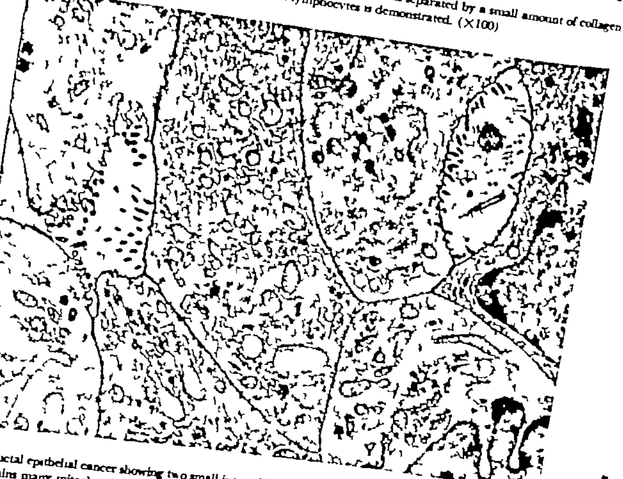


Fig 5 Ductal epithelial carcinoma showing sheets of neoplastic cells separated by a small amount of collagen. Stromal infiltration with lymphocytes is demonstrated. (×100)



uctal epithelial cancer showing two small intercellular spaces lined by short microvilli. The cytoplasm
ains many mitochondria and a few strands of rough-surfaced
mic reticulum. (×7,500)

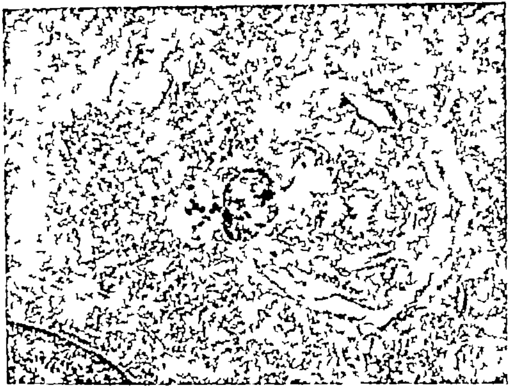


Fig. 7 Ductal epithelial cancer showing positive reaction for acid phosphatase in the lysosomes. Notice the lack of the reaction in the Golgi apparatus. (×30,500)

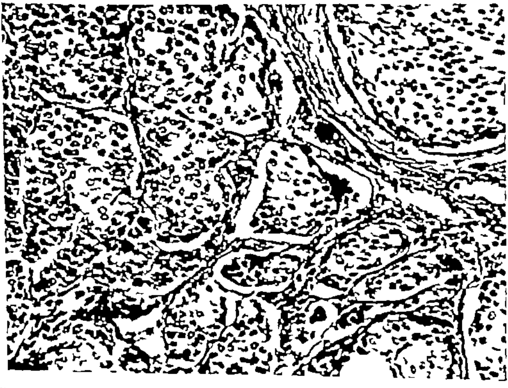


Fig. 8. Infiltrating ductular carcinoma showing groups of tumor cells in nodular patterns separated by delicate stroma. Notice the pale staining cytoplasm of the neoplastic cells. (×250)

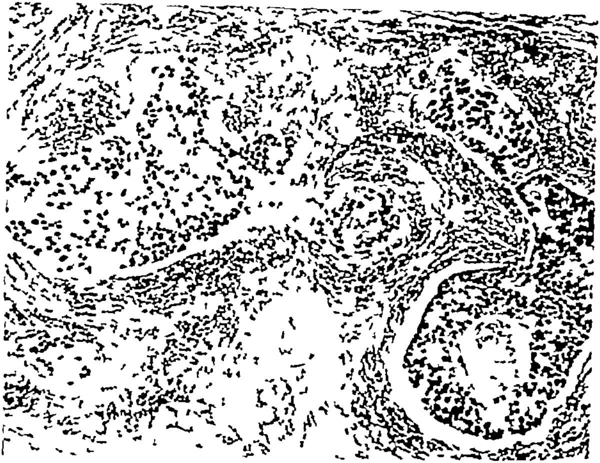


Fig 5. Ductal epithelial carcinoma showing sheets of neoplastic cells separated by a small amount of collagen Stromal infiltration with lymphocytes is demonstrated. (×100)

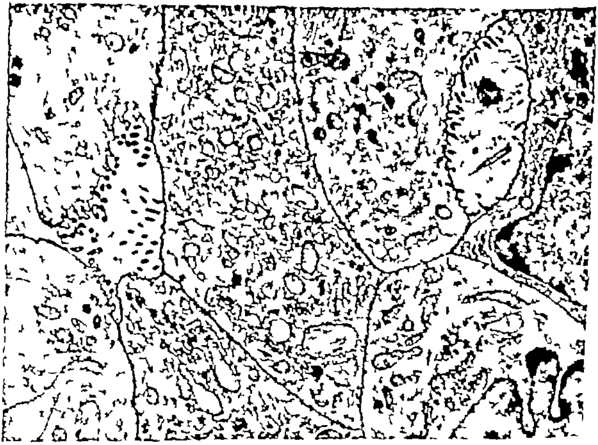


Fig 6 Ductal epithelial cancer showing two small intercellular spaces lined by short microvilli. The cytoplasm contains many mitochondria and few strands of rough-surfaced endoplasmic reticulum. (×7 500)

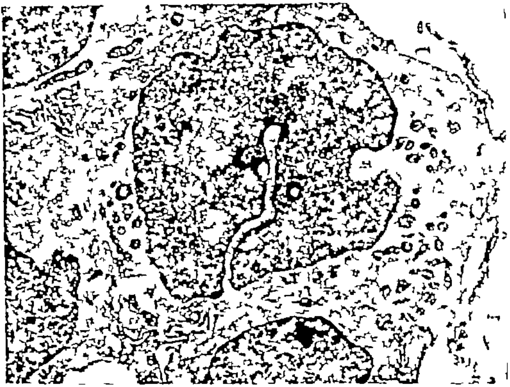
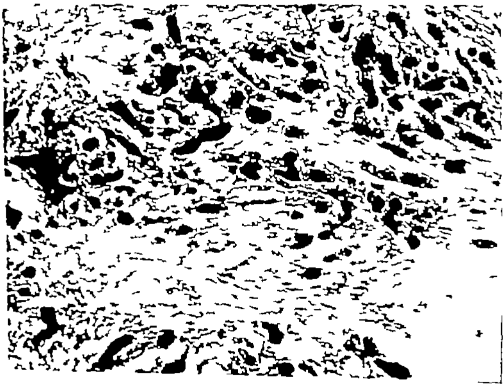


Fig. 10. Infiltrating ductular carcinoma with large nuclei and little cytoplasm. The nuclei contain patche of heterochromatin throughout the nucleoplasm. Cellular cohesion is minimal and special types of cell to cel attachments are limited to the surface area. (×25,000)



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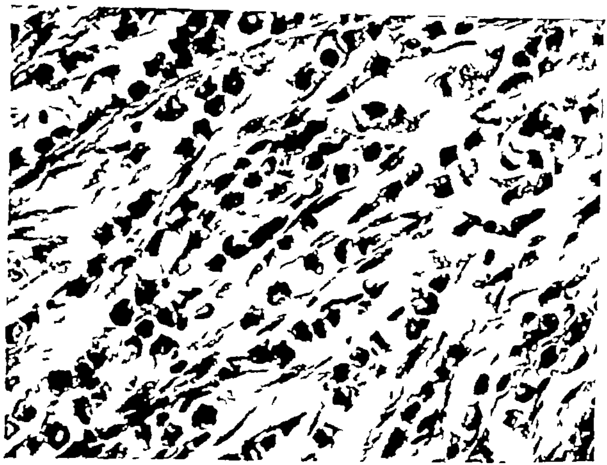

Fig 9. Infiltrating ductular carcinoma showing infiltration by singl cells in delicate reticular stroma The neoplastic cells show loss of cellular cohesion and the individual tumor cell is surrounded by an empty space (×400)

seems important. The methods for cellular differentiation used in our study are tedious, time-consuming and expensive and for this reason we had to limit our study to fewer cases (37) than in a simple histologic review of surgical material. The limited number of cases in our study decreased its statistical significance. Nevertheless, it showed that the three types of carcinoma of the breast proposed by us differed in the frequency of metastasis since the number of lymph nodes involved in metastasis was 101 out of 313 nodes or eight of 14 cases of ductular carcinoma three out of 216 nodes or one out of 11 cases in ductal carcinoma and one out of 163 nodes or one in 12 cases of myoepithelial carcinoma. If the presence of axillary metastasis is used as a prognostic criterion, one would expect that the ductular carcinomas would be associated with a lower survival rate than ductal or myoepithelial carcinoma Since ductular epithelial cells are the only cells responding to the hormonal influence associated with pregnancy [11] one may rightly assume that ductular carcinoma is more dependent upon sex hormones than the other two types This of course does not *imply that they are produced by hormonal* dyscrasias but it explains their age incidence and the frequent observation of multifocal or bilateral origin. Since the male breast lacks ductular formation,[1] *it also explains why ductular carcinoma is* restricted to women

The presence of the enzymes ATPase and IDPase on the plasma membrane of myoepithelial carcinoma and their absence in ductal and ductular carcinomas confirm our previous suggestion that the myoepithelial cell is the cell of origin of scirrhous carcinoma [9] Alkaline phosphatase, a prominent enzyme in myoepithelial cells, disappears when these cells undergo malignant transformation. However alkaline phosphatase remains positive within the blood vessels of the neoplasms and this fact may explain the false positive results of purely chemical analysis for these enzymes. The distribution of acid phosphatase in ductular carcinoma needs further study

No follow-up studies were done on those cases because of the small number studied by these techniques and because the study was started within the last few years which makes the statistical significance very doubtful The lymph nodes and the

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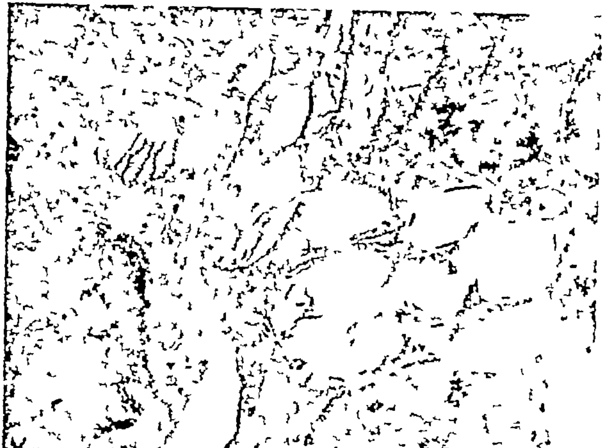

Fig 12. Infiltrating ductular carcinoma featuring free acid phosphatase in the cytoplasm and also in areas of heterochromatin of the nucleus. Notice the absence of the reaction from the mitochondria and from lipid droplets. (×23,000)

degree of their involvement with tumor however were recorded in each case and the total number of lymph nodes with or without tumor in all cases was counted The data were included in this paper because of the reported lower percentage of five year survival in patients with breast cancer in which the axillary lymph nodes were involved at the time of surgery

SUMMARY

Carcinoma of the breast was classified according to its cellular origin into myoepithelial, ductal epithelial, and ductular epithelial carcinomas The light microscopic appearance, the ultrastructural cytology and the enzyme pattern of the three types of cancer were described. The study showed that ductular carcinoma of the breast was associated with a higher incidence of metastasis than the other two types. The possible dependency of ductual carcinoma on hormonal influences was discussed.

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TABLE I. *Individuals Tested by Age and Sex. Number and Per cent with Counts under 11 Megakaryocytes (Mk) per 10 ml*

Age Group	Males			Females			Total Both Sexes		
	Number Tested	Under 11 Mk/10 ml		Number Tested	Under 11 Mk/10 ml		Number Tested	Under 11 Mk/10 ml	
		No	%		No	%		No	%
20–29 yrs.	5	3	60	5	4	80	10	7	70
30–39	6	1	17	5	3	60	11	4	36
40–49	6	2	33	5	4	80	11	6	55
50–59	9	7	78	6	5	83	15	12	80
60–69	5	4	80	7	6	86	12	10	83
70–79	6	5	83	4	3	75	10	8	80
80+ yrs	8	8	100	3	3	100	11	11	100
Total	45	30	67	35	28	80	80	58	72

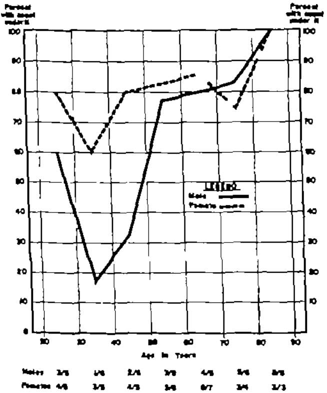

FIG. 1 Megakaryocyte counts of 80 normal individuals. Percentage with counts under 11 Mk/10 ml by age and sex, 45 males and 35 females.

had counts greater than 10 megakaryocytes per 10 ml. A chi-square test showed the difference to be significant with P < 02. This pattern was not obvious among female subjects. Eighty-five per cent of females aged 60 years or older had counts of less than 11 megakaryocytes per 10 ml, and at least 75 per cent of females under 60 years of age had similarly low counts (Table II). The data for the males were further analyzed to determine whether the proportion of low counts (under 11 megakaryocytes per 10 ml) followed linear trend with respect to age over the seven decades studied. A positive slope was found to exist and to be statistically significant with P < 01 (Fig 1) The pro-

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Circulating Megakaryocytes: A Quantitative Observation in Normal Subjects by Age and Sex

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Megakaryocytes are normal constituents of the peripheral blood and their number usually varies from individual to individual.[1] In a previous study however, it was observed that among non-cancerous males, the average megakaryocyte count tended to decrease with increasing age.[2] No such tendency was noted among females. The subjects were hospitalized patients and represented a limited number of cases in the various age groups studied. The present study was designed to further investigate the relationship between megakaryocyte counts and age and sex in "normal" individuals.

Methods and Materials

At least two 10 ml samples of peripheral blood were drawn from the antecubital veins of 80 normal individuals. All blood specimens were obtained in the morning to avoid diurnal variations. Duplicate samples from each person were taken at intervals of not less than four weeks. A sample of 45 males and 35 females was selected with approximately equal numbers in each decade beginning with age 20 (Table I). The majority of subjects were hospital staff volunteers (technicians, nurses, house staff). Individuals in the eighth and ninth decades resided in the Riverview Nursing Home, an affiliation of Beekman-Downtown Hospital. No person was included in the study if there was a medical history indicating a need for hospitalization and or any type of treatment six months prior to examination, with the following exception: some in the older age groups with compensated cardiovascular disease requiring only maintenance doses of digitalis and/or diuretics.

All blood samples were collected with heparin as the anticoagulant and processed in the same manner as previously described.[2] Following isolation by the dextran rapid sedimentation method,[4] nucleated cells were collected on membrane filters (five micron pore size) and then washed continuously with saline to insure an even distribution. All filters were fixed with Carnoy's solution and then placed on glass slides according to the method of Chang and Russell. The cells were stained by Papanicolaou technique, carefully screened by cytotechnologists and finally reviewed by a pathologist. Megakaryocytes were identified without any appreciable difficulty.

Results

Since more than 60 per cent of subjects from the previous study had counts of 10 megakaryocytes or less per 10 ml, it was decided that any count above this number could be considered high. Each of the age groups was arbitrarily divided into those individuals with counts greater than 10 and those with counts of 10 or less megakaryocytes per 10 ml. Since there was reasonable correspondence in the numbers of megakaryocytes counted in repeated samples obtained from a single subject, only the average number of megakaryocytes per 10 ml for each individual was used. Only two subjects were observed for whom one sample contained a count greater than 10 megakaryocytes per 10 ml while the other was below this figure. These two cases were excluded from the study.

The proportion of individuals exhibiting counts of 10 megakaryocytes or less per 10 ml by age group is tabulated in Table I. It is apparent that from the sixth decade on almost all subjects consistently exhibited counts below 11 megakaryocytes per 10 ml. Approximately 90 per cent (17 out of 19) of the males 60 years of age or older had a count under this critical number while 50 per cent (13 out of 26) of the males less than 60 years of age

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§ Director of Cancer Research & Control, New York City Health Department (Deceased June 16, 1970).

cytes per 10 ml. The significance of these counts is discussed.

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TABLE II Megakaryocyte Counts of 80 Normal Individuals tested by Age-Group and Sex. Number and Percent with Counts under 11 and 11 or More Megakaryocyte (MK) per 10 ml

Megakaryocyte count (MK/10 ml)	Males						Females						Total Both Sexes					
	Under 60 years		60 years and over		Total		Under 60 years		60 years and over		Total		Under 60 years		60 years and over		Total	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	%
Under 11	13	(50%)	17	(89%)	30	(67%)	16	(76%)	12	(86%)	28	(80%)	29	(62%)	29	(88%)	58	(72%)
11 and over	13	(50%)	2	(11%)	15	(33%)	5	(24%)	2	(14%)	7	(20%)	18	(38%)	4	(12%)	22	(28%)
Total	26	(100%)	19	(100%)	45	(100%)	21	(100%)	14	(100%)	35	(100%)	47	(100%)	33	(100%)	80	(100%)

portion of low counts in male subjects above and below the age of 60 in this study agrees with the results for the control patients (non-cancer patients) in a previous report.[3]

A striking observation is the high proportion of both male and female subjects in the 30 to 39 age group having megakaryocyte counts of more than 10 per 10 ml. Though this could be due to "chance variation," the possibility of having a physiologic significance cannot be entirely ruled out. The influence of menses in female subjects in this age group was entertained; however, other studies[4] have indicated that there is no consistent change in the number of circulating megakaryocytes during the various periods of the menstrual cycle.

DISCUSSION

Based on the techniques used in this investigation, it was decided that 10 megakaryocytes per 10 ml could be considered as the limit of high normal. Low counts were a consistent finding in all subjects in the seventh decade or older, though females as a group tended to have counts below 11 megakaryocytes per 10 ml. Males below the age of 60 showed considerable variation in the proportion of low counts. The results of this study utilizing healthy non-hospitalized subjects, were in general agreement with findings in the non-cancer patients studied in a previous investigation.[3] It can be generalized that counts higher than 10 per 10 ml in females of all ages or in males 60 years of age or older may be considered unusual. Although we do not suggest that high numbers of circulating megakaryocytes indicate a pathologic process, the possibility that high megakaryocyte counts in these age-sex related groups may have a physiologic basis, cannot be summarily dismissed. It should be stressed, however, that one must carefully weigh the limited contribution that this test can offer as a diagnostic tool against the amount of time required to perform this tedious and somewhat costly examination.

SUMMARY

Megakaryocyte counts were performed on 80 apparently normal subjects (45 males and 35 females) to determine the range of values on an age-related basis. Individuals were selected to provide approximately equal numbers in each of the decades from the third through the ninth. Ten megakaryocytes per 10 ml was designated as the limit of high normal. Low counts were a consistent finding in all subjects in the seventh decade older. Males below the age of 60 showed con able variation in counts while females as tended to have low counts below 11

The Committee on Registration and Licensure approved several changes in the procedure and registration prerequisites for the C.T (I.A.C.)-Registry Examination. The following changes were approved

(1) Cytotechnologists from countries in which a national licensure examination or national registry examination for cytotechnologists exists, may apply for the I.A.C. Registry if they have previously successfully completed the national examination. Documentary proof is required.

(2) Of the two letters of recommendation which are required for the admission of the cytotechnologist to the I.A.C. Registry Examination, at least one letter must be from a Fellow or Member of the International Academy of Cytology if there is a Fellow or Member of the I.A.C. in the country of the applicant.

(3) Two-year full-time experience in cytotechnology is required (after the completion of the cytotechnology training) before the cytotechnologist may apply for the C.T (I.A.C.) Registry Examination

(4) For cytotechnologists who apply for the I.A.C. Registry Examination after July 1 1971 the following registration fee structure will apply The application fee will be $25.00 for cytotechnologists who were trained by a Fellow or Member of the International Academy of Cytology or who currently work for a Fellow or Member of the I.A.C. For all others the fee will be $50 00 (U S. currency) For applicants who submit their applications prior to July 1 1972 the fee will be $25 00 (U S. currency)

(5) The International Academy of Cytology will conduct the C.T Registry Examinations in cooperation with national or regional societies of cytology if the need arises This cooperative venture with national and regional societies was approved to assure that qualified cytotechnologists may take the I A C. Registry Examination without major travel expenses

(6) The next I A.C.-Registry Examination will be given on June 17 1972 at the Vienna Intercontinental Hotel, Vienna, Austria following the *Second International Tutorial on Clinical Cytology* (June 11–17 1972) The deadline for application (receipt in Chicago) is February 1 1972

The President of the Academy has appointed a Registrar who will be responsible for the initial evaluations of the applications and for the evaluation of the results of the examinations Catherine M Keebler CT (ASCP) CT (IAC) was appointed Registrar

The Committee on Registration and Licensure of the International Academy of Cytology approved also the following changes in the procedure and the requirements for CERTIFICATION OF LABORATORIES OF CYTOLOGY

(1) As of January 1 1974 laboratories applying for certification by the International Academy of Cytology must employ at least one cytotechnologist who has successfully passed the C.T (I.A.C.)-Registry Examination.

(2) As of January 1 1975 laboratories applying for certification must be headed by a Member or Fellow of the International Academy of Cytology

Information about registration of cytotechnologists and about certification of laboratories of cytology may be obtained from

The International Academy of Cytology
Committee on Registration and Licensure
5841 Maryland Avenue HM #449
Chicago, Illinois 60637 U.S.A.

THE INTERNATIONAL ACADEMY OF CYTOLOGY

Committee on Registration and Licensure

The following cytotechnologists have taken and successfully passed the Registry Examination for cytotechnologists, and are entitled to use the abbreviations *C T (I A C)* following their names

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Miss Ester Helena Lehto, C.T (I.A C.) Reg No 19
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Tampere Finland

Canadian Society of Cytology—Societe Canadienne de Cytologie

The next meeting of the Canadian Society of Cytology will be held jointly with the Canadian Association of Pathologists in Saskatoon, Saskatchewan, June 20 to June 23. The Program will include a Panel on "The Value of Gynaecological Cytology—A Current Assessment" with the participation of Doctor H. K. Fidler Vancouver Doctor J P A. Latour Montreal Doctor G H. Green, Auckland, New Zealand and Doctor A. Phillips, the National Cancer Institute of Canada The Ortho Guest Lecture will be delivered by Doctor Zuher M. Naib, of Atlanta, Georgia.

Further information may be obtained from Doctor R. H Curry Secretary Treasurer Canadian Society of Cytology Ottawa Civic Hospital, Ottawa, Ontario.

Cours De Cytologie Vaginale Hormonale

Un enseignement de Cytologie Vaginale Hormonale sera donné du 15 au 19 novembre 1971 à la Clinique Gynécologique et Obstétricale de l'Université de Liège, Belgique, sous le patronage de la Fédération Européenne des Sociétés de Cytologie.

Il comprendra

—les matins, de 9 h. 30 à 12 h. 30, des exposés théoriques par les Professeurs BETZ, BOURG, FERIN et PUNDEL et les Docteurs CREPET GOMPEL, LICHTFUS, MARSAN PELZER, WACHTEL et WURCH

—les après-midi, de 15 h à 17 h. 30 des travaux pratiques.

Il sera suivi, le 20 novembre 1971 par la réunion commune des Sociétés Française et Belge de Cytologie Clinique, qui sera consacrée aux "oestro-progestatifs de synthèse et cancer cervical" avec des exposés des Professeurs DE BRUN et FERIN

Pour détails et inscriptions, s'adresser au Docteur Andrée PELZER, Laboratoire de Cytologie, Clinique Gynécologique et Obstétricale de l Université de Liège, 81 boulevard de la Constitution, B–4000–Liège–Belgique.

The European Federation of Cytology Societies

The European Federation of Cytology Societies (E.F C.S) will sponsor the Second European Cytology Congress in Budapest, Hungary October 3-5 1972
The Main Topics of the Congress will be

(1) Cytology as technic for early cancer detection (screening method)
(2) New advances in cytologic assessment of endocrinologic disorders
(3) Advantages and limitations of the aspiration biopsy technic
(4) New methods of diagnostic cytology

The program will include invited lectures, proffered papers, round table discussions and demonstration of slides Lecture time of proffered papers is limited to ten (10) minutes.

The *official languages* of the Congress are English, German and French.

The Registration Fee is US $35.00 for cytotechnologists the fee will be $20 00 for students $15 00 and for accompanying family members also $15 00

Further details about the European Cytology Congress may be obtained by writing to Dr Gabor Sassy Dobray Janos Hospital Diosarok U 1 Budapest XII Hungary

The following Executive Council of the European Federation of Cytology Societies (E.F C.S.) was elected on May 23, 1971 (for the years 1971/72)

President Dr G Sassy-Dobray Budapest, Hungary
President Elect Dr J Zajicek, Stockholm, Sweden
Past President Dr E. Wachtel London, England
Secretary General Dr J Jenny Zürich Switzerland
Secretary of the Congress Dr L. Döbrössy Budapest, Hungary
First Treasurer Dr P Lopes-Cardozo, Leiden, Holland
Second Treasurer Dr W Ebert, Jena, DDR-Germany

All communications pertaining to the E.F C.S should be directed to

Dr Jacques Jenny
Secretary General E.F C.S.
Plattenstrasse 51
CH-8032 Zürich, Switzerland

Please direct also communications about the following meeting to Dr Jenny

The THIRD WORKSHOP SESSION ON CLINICAL CYTOLOGY jointly sponsored by the Swiss, German and Austrian Societies of Cytology (in German language) will take place in Wengen, Switzerland April 8-15, 1973 The main topics will be uterine carcinomas and cytology of effusions.

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LETTERS TO THE EDITOR

Antioxidant Inhibition of Stain Fading and Mounting Medium Crazing

To the Editors:

We have previously noted[2] that microscopic slides prepared with certain synthetic mounting media characteristically develop cracking or crazing patterns which increase with time, as indicated by the development of annual rings. This progressively diminishes the usable area of the smear and, in addition, a wide zone of severe fading develops in the stained areas immediately adjacent to the crazing, so that old slides must be periodically reprocessed. The following observations shed further light on crazing and fading and suggest a method for avoiding it.

TABLE I. *Inhibition of fading and crazing in Wright-stained blood smears by an antioxidant* (1% (w/v) 2-6-di-*tert*-butyl-*p*-cresol)

Mounted in 1963 with	Fading 1964	Fading 1971	Crazing 1971
Gum Damar	+*	+	0
Gum Damar plus antioxidant	0	0	0
Permount	+*	++*	+*
Permount plus antioxidant	0	0	0

* Generalized loss of stain intensity and marked fading of the basophilic component of stain.

Fading has not progressed since 1964.

New peripheral zones of severe fading adjacent to areas of mounting medium crazing.

Eight annual bands present.

We have re-examined a number of Wright-stained blood smears originally prepared and mounted for the study of inhibition of fading of microscopic preparations by an oil-soluble antioxidant (2-6-di-*tert*-butyl-*p*-cresol).[1] In extension of the original observations, smears mounted in either gum damar or Permount with added antioxidant still retained their original, precise, brilliant stain after eight years. It is remarkable, however, that although control smears mounted with regular Permount now show the expected crazing with annual bands, and now have an additional zone of peripheral fading, *no crazing and no peripheral fading has developed in slides mounted with Permount containing antioxidant*. The observations are summarized in Table I. In addition, a number of Papanicolaou-stained cellular smears prepared with Permount plus antioxidant are unfaded and without crazing after a relatively shorter period of time (three years).

The findings are consistent with the propensity of unsaturated high polymers to undergo crystallization with oxidation.[3] Evidently, since the antioxidant is inhibitory, both progressive fading in stained microscopic slides and crazing of mounting media are effects of oxidation.

Summary

An oil-soluble antioxidant (e.g., 2-6-di-*tert*-butyl-*p*-cresol) in resinous mounting media inhibits both fading and crazing of histologic and cytologic specimens.

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The Use of the Crushing Condenser for Photometric and Other Cytologic Studies on Histologic Sections

To the Editors:

Accurate measurement of the stain content of single cells with the Deeley integrating microdensitometer generally requires that the cells be flattened, e.g., with the crushing condenser[1] which is supplied with the commercial model (Barr and

focal plane but also to ensure that any unstained material that is visible immediately adjacent to the object lies only in the plane of the latter and not above or below this plane. Thereby the object and the material surrounding it are converted into thin "sandwich thus serving substantially to reduce possible errors in the microspectrophotometric technique[1]

Technical problems associated with the use of sections for microspectrophotometry have been discussed by Sandritter *et al* They describe method of squashing sections under layer of plastic foil between two slides. Use of the crushing condenser however offers rather more flexibility in that small selected areas can be flattened under direct microscopic observation

Cytologic features such as sex chromatin may be more readily amenable in thick sections after flattening than in thin permanently-mounted sections where many of the nuclei are sliced or overlapping Assuming good fixation and the use of suitable stains, cytologic squash technique may in effect be applied to histological material (Fig 2)

Acknowledgments

I thank Dr M. H. Bennett, director of the Pathology Department, for his kind cooperation and Mr J F Howlett for technical assistance. This work was supported by a grant from the Cancer Research Campaign

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3. Sandritter W Pilny J Novakova, V and Kiefer G Zur Problematik der Gewebspraparation fur cytophotometrische Messungen. Histochemie 7 1 7 1966.

Stroud Ltd Glasgow type GN2) Permanently mounted histologic sections are therefore not suitable for use with this technique, but on the other hand I have found that if sections are mounted on coverslips in water to which a drop or two of glycerol has been added and covered by cellophane (the usual method of mounting smears[3]) the cells can be adequately flattened with the crushing condenser for measurement of Feulgen stain content. At the same time they can be spread out so that previously overlapping nuclei can often be isolated for measurement, although this is not so easy to accomplish as with smears

Using sections rather than smears, the location of the cells that are being measured can of course be determined in relation to the histologic pattern of the tissue the possibility of using material from paraffin blocks may allow measurements to be made on cases from which no other material is available.

If the sections are of an appropriate thickness (e.g. 50–100 microns) sliced nuclei will be relatively infrequent fragments of nuclei can usually be recognised as such and, in any case, if it is desired to establish the modal value of a population of premalignant or malignant cells, will probably fall well below the modal range A few nuclei with small portions sliced off may go undetected and could give rise to a shift in the mode to the left (the significance of this will, of course be reduced if the cells are being compared with control cells whose mode is similarly shifted) In practice, I have found good agreement between measurements on sections and smears from the same cases (Fig 1)

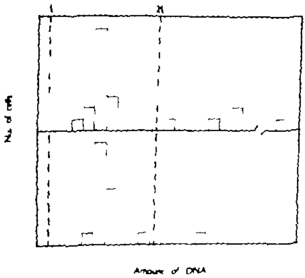

FIG. 1 DNA values of interphase epithelial cells from a well-differentiated malignant teratoma of the testis (patient aged 30) Above from a smear fixed by freeze substitution[4] below from a 50 micron section. Both were stained by the Feulgen technique. Each distribution shows a mode in the triploid region (I = mean value of leucocytes present in the tissue 2I = twice this value)

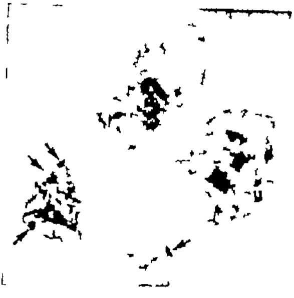

FIG 2. Epithelial nuclei from a 50 micron Feulgen-stained section of a carcinoma of the colon (female aged 65) flattened by the crushing condenser Arrows indicate three sex chromatin bodies in one nucleus and one in a fragment of a nucleus previous studies on orcein squash preparations showed three bodies in many of the nuclei ×2200

The flattening and spreading out of the cells is aided by moving the stage sideways while pressure is applied by raising the condenser Cells near the edges of the section are usually more amenable to this treatment, but it is quite feasible to remove small pieces of tissue under a dissecting microscope from selected regions lying within the boundaries of the section and measure these separately at the same time the cells bordering the "holes in the section thus produced can be spread with greater ease Where there is little cytoplasm, it may be impossible to isolate many of the nuclei, but a representative sample can usually be made accessible for measurement without difficulty Carcinomas in situ and dysplasias of the cervix generally provide suitable material

When the flattening is sufficient to bring the object wholly within the depth of focus of the objective conditions are suitable for measurement. Sometimes unstained material is visible outside the object but within the scanned area this may give rise to an error through the loss of light by scattering which however can be minimised by including a similar amount of the material in the subsequent blank measurement instead of making the latter in a region of the coverslip clear of the section Flattening with the crushing condenser should be sufficient not only to bring the object within the neous in the measured property Electronic sorting of cells was first announced by this author in 1965 Cells suspended in an aqueous medium pass through a sensor where volume is measured, subsequently the suspension jets into air where it breaks into droplets, some of which contain cells. The droplet containing selected cell is electrically charged and deflected into a receptable. Cells are analyzed at the rate of 2000 sec and can be sorted to yield separated fractions containing less than 10 contamination by unwanted cells Separation of cell requires 0.1 msec of time and deflection of corresponding amount of flowing liquid I studies on the colony-forming units in mouse bone marrow 10^6 cells of single volume class has been isolated in a single 90-min run Presently the electronic cell sorter simultaneously sorts into independently adjustable volume ranges and waste receptacle This system has been applied to the analysis of human leukocytes, to cell types within mouse bone marrow to colony forming units in mouse bone marrow and to the development of leukemia in AKR mice In the original paper the possibility of extension to optical and multiple sensors was discussed such development is currently underway using the optical sensor system of Van Dilla *et al*

In 1967 Kamentsky *et al* described another type of cell sorter which was modification of their previously described cell analysis system This system sorts cells according to the ratio of ultraviolet light absorption to light scattered by the cell If the ratio of these signals falls within predetermined range motor-driven syringe is activated to draw an increment of flowing liquid containing the cell into side channel Because of the uncertainty of cell transit time from the sensor to the point of separation and the inertia of the motor-driven syringe the rate of separation is quite low requiring 3.0 msec per cycle During this interval, the cell suspension is diverted to flow into the syringe Apparently this volume of liquid usually contains several cells in addition the selected one consequently the separated cell suspension is relatively inhomogeneous This device has been applied to cells obtained by vaginal wash of patient having carcinoma of the uterine cervix

Hulett *et al* recently announced an extension of the droplet scheme described above in which cells are electronically selected according to their degree of fluorescence and subsequently captured into charged liquid droplets and deflected into collection vessel Enrichment of antibody forming cells has been obtained from mouse spleen cells made fluorescent by the fluorochromatic technique of Rotman and Papermaster

Principle of Electronic Sorting

In order to discuss the limitations and potentials of electronic sorting, more detailed description of our technique follows As shown in Figure 1 cells suspended in an electrically-conducting liquid enter the volume sensor and droplet generator through an inlet tube under a pressure of approximately two atmospheres Within the generator the suspension passes through an orifice in nonconducting material wherein the volume of each cell is electronically measured in 50 µsec by the Coulter principle The suspension then passes through an orifice of 50 µm diameter in platinum electrode and emerges into open air as a free liquid jet of about 45 µm diameter Ultrasonic vibrations produced by piezoelectric crystal are transmitted to the cell suspension by a plastic coupling rod The resulting small pressure fluctuations in the suspension cause the velocity of the emerging jet to vary periodically producing regular bunching of the jet Because cylindrical jet is unstable these disturbances grow by surface tension forces until the liquid

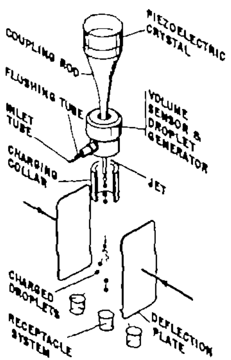

Fig. 1 Electronic cell sorter Suspended cells pass through an orifice within the volume sensor and droplet generator wherein volume is electronically measured. The cell suspension then jets into air and breaks into 100 µm droplets so that one cell is found in every 33 droplets. Droplets containing selected cells are charged and deflected into receptacle.

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AUTOMATED CYTOLOGY

A Symposium by Correspondence

Chapter VII Sorting or Marking the Prescreened Cells

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Opening Remarks

DR. FULWYLER

Electronic Sorting of Cells

Quantitative measurement of cellular properties by electronic or optical methods as cells in aqueous suspension flow past a sensor is a relatively recent development. The first such device (in 1956) was the Coulter counter, which is able to measure cell volume. In 1965 Kamentsky *et al.* developed a flow-through system in which absorption of ultraviolet light by a cell traversing the beam is a measure of nucleic acid content; additionally, the light scattered is related to cell size. Van Dilla *et al.* recently described a laminar sheath-flow microfluorometric system using an argon-ion laser (wavelength 488 nm) to excite the fluorescence of cells stained with a fluorescent dye. This system has given quantitative measurement of cell DNA content. Mullaney *et al.* have used a helium-neon laser and the flow system described by Van Dilla *et al.* to study the small-angle forward scattering of cells in which the scatter signal is related to cell volume.

Electronic sorting

Sorting of cells is an outgrowth of the desire to isolate for further study a subpopulation homoge-

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This work was performed under the auspices of the U. S. Atomic Energy Commission.

applications it might be desirable to catch the droplets on filter paper or moving tape and perhaps to mark the position for later location.

The ability to isolate a subpopulation of cells stimulates development of new sensing systems because it makes possible the direct correlation of relatively abstract electronically or optically measured properties with the more traditional morphologic criteria. Indeed, development of multiple sensor systems and corresponding development of biochemically-specific staining techniques, including fluorogenic enzyme assay techniques and fluorescent antibody-antigen techniques, may define new type of "morphology" in which cell types are defined in terms of several quantitatively measured biochemical and functional properties such as RNA-DNA ratios, presence of specific enzymes, reaction with specific antibodies, etc. The ability to sort cells will be especially important in determining the significance of multiple-parameter combinations and will provide guidance in development of criteria to uniquely identify a specific type of cell.

Rel Cytopathology

Electronic sorting can play an important role in the application of flow-system analysis to automated cytopathology. Sorting can provide the means by which development of sensors and various staining or labeling methods can be optimized to uniquely characterize cells of interest. Until adequate confidence in automatic analysis is attained, sorting will be desirable to permit visual verification of abnormal cells.

One can imagine an integrated system in which sample of suspended cells flows past several sensors to measure different properties. The signals generated are processed, compared with predetermined criteria, and assigned to one of perhaps four categories, depending on the degree of abnormality. Cells of each category would be separated and collected on moving tape, which would be marked to facilitate subsequent relocation and visual examination. The number of cells in the gross sample and in each category would be recorded along with a sample identification number. As the sample is analyzed, frequency distributions of selected characteristics would be produced and recorded on magnetic tape, on printed tape, on IBM cards for computer handling or plotted for immediate use. Such data may reveal prepathologic conditions by making apparent a deviation from the normal pattern. A system of this type potentially combines the advantage of rapid, automatic prescreening with the confidence of visual examination of sorted and concentrated abnormal cells.

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Dr KAMENTSKY

Marking or Sorting of Prescreened Cells with Special Consideration of Fluidics

The screening of cytologic material is often done by hierarchy of two or more levels of screening. The prescreening philosophy employed by cytotechnicians and pathologists for later verification higher screening level can also be employed by a hierarchy of different instruments with different screening discriminations and processing rates, or the instruments may prescreen directly for the cytotechnician or pathologist. This prescreening can be performed on a sample basis, whereby the instrument indicates whether given sample should be considered by column breaks into droplets of approximately 100 µm diameter Droplets are uniform in volume and spacing and are produced at the frequency of piezoelectric crystal oscillation (about 70 000/sec) In this way each cell in the suspension is isolated in a single liquid droplet. As droplets detach within the charging collar a voltage applied between the generator and the collar will induce a charge on the forming droplet. Once detached, the droplet retains its charge and passes into a static electric field between two deflection plates wherein charged droplets are deflected to one side or the other according to the sign and magnitude of their charge Deflected droplets are collected in beakers or other collection system.

The sequence of events in sorting can be briefly summarized As a cell traverses the sensing orifice, it produces an electric pulse with an amplitude directly related to cell volume which is then electronically analyzed. If the pulse amplitude falls within a preselected range a charging pulse will be applied to the charging collar after a delay of 200 µsec during which the cell moves from the sensing region to the point of droplet formation. The charging pulse is flat-topped and is maintained for approximately 100 µsec during which seven droplets will form and detach one of these contains the selected cell The charged droplets are then deflected into a collection vessel to complete separation Uncharged droplets pass undeflected into a waste receptacle Since droplets are being produced at the rate of 70 000/sec and cells pass through the sensing orifice at the rate of 2000 sec, on the average one cell is found in 35 droplets and the remaining 34 droplets contain no cells Thus, the probability of finding two cells in a group of seven droplets is quite small and single cells can be cleanly isolated from the mixture A more detailed description of this device including electronic systems, has been published elsewhere

Limitations and Potentials

Automatic cell analysis in flow systems requires that the cells be singly dispersed Clumps of two or more cells stuck together will produce an erroneous signal, which may be important if one is seeking to detect a few large cells among many For some purposes, the ability to sort cells for later examination will lessen the detrimental effect of such clumps All flow systems and associated separation systems have a fundamentally limited rate of analysis because cells must traverse the sensing region one at a time, and a finite amount of time (approximately 60 µsec) is required to analyze each cell In order to minimize the coincidence of two or more cells in the sensing region, the suspension must be diluted to the point that the sensing region is void of cells 90 per cent of the time Consequently the maximum rate of analysis is a few thousand per second, depending on the time a particle remains in the sensing region and on the rate limitations of the associated electronics.

When used as a preparative tool to sort a large number of cells for subsequent study the sorting rate depends on the analysis rate and on per cent of the original sample which falls within the selected range. The droplet method of sorting is an inertia-free system in that no parts move in separating a cell. It is truly electronic, as only electric charge is used to control liquid flow The amount of liquid which is deflected in separating one cell is quite small (about [illegible] × 10[illegible] liter in seven droplets)

The concept of electronic sorting is broad and can be extended to any sensor system which can rapidly measure a cell property as do the fluorescence and light scattering techniques discussed above. Also, because of the electronic nature of the droplet separation scheme, many existing electronic techniques may be applied. For multiple-parameter systems, the signals from several sensors may be processed in a variety of ways to obtain ratios, differences, sums, overlapping ranges, etc and the decision to separate a cell can be made contingent upon the satisfaction of several criteria In the present volume-measuring cell sorter an anticoincidence circuit is incorporated which will avoid separation if the selected cell is followed or preceded closely in time by an unwanted cell such that it could not be cleanly separated Because the process of separation is associated with a particular cell only through the sensor the degree of homogeneity of a sorted subpopulation of cells is primarily determined by the resolution of the sensor system. For certain applications of sorting viability of the separated cells is essential This has been demonstrated for the droplet system and would seem to be compatible with the fluidic technique.

Because the degree of deflection in the electrostatic field is proportional to droplet charge, it is possible to sort simultaneously into several classes. Currently the volume separator sorts into two classes plus the waste receptacle Simultaneous separation into four classes plus waste has been obtained by giving droplets of class I a charging pulse of +30 V amplitude class II +60 V class III −30 V and class IV −60 V thus, classes I and II are deflected different distances to one side and caught in separate vessels and classes III and IV are deflected to the other side. Sorting into six or more classes seems feasible Collection of the deflected droplets can be done in several ways they may be caught in a beaker partially filled with a compatible medium, or for some applications it might be desirable to catch the droplets on filter paper or moving tape and perhaps to mark the position for later location

The ability to isolate a subpopulation of cells stimulates development of new sensing systems because it makes possible the direct correlation of relatively abstract electronically or optically measured properties with the more traditional morphologic criteria. Indeed, development of multiple sensor systems and corresponding development of biochemically-specific staining techniques, including fluorogenic enzyme assay techniques and fluorescent antibody-antigen techniques, may define a new type of "morphology" in which cell types are defined in terms of several quantitatively measured biochemical and functional properties such as RNA DNA ratios, presence of specific enzymes, reaction with specific antibodies, etc. The ability to sort cells will be especially important in determining the significance of multiple-parameter combinations and will provide guidance in development of criteria to uniquely identify specific type of cell.

Role in Cytopathology

Electronic sorting can play an important role in the application of flow-system analysis to automated cytopathology. Sorting can provide the means by which development of sensors and various staining or labeling methods can be optimized to uniquely characterize cells of interest. Until adequate confidence in automatic analysis is attained, sorting will be desirable to permit visual verification of abnormal cells

One can imagine an integrated system in which sample of suspended cells flows past several sensors to measure different properties. The signals generated are processed, compared with predetermined criteria, and assigned to one of perhaps four categories, depending on the degree of abnormality. Cells of each category would be separated and collected on moving tape, which would be marked to facilitate subsequent relocation and visual examination. The number of cells in the gross sample and in each category would be recorded along with sample identification number. As the sample is analyzed frequency distributions of selected characteristics would be produced and recorded on magnetic tape, on printed tape, on IBM cards for computer handling, or plotted for immediate use. Such data may reveal prepathologic conditions by making apparent deviation from the normal pattern. A system of this type potentially combines the advantage of rapid, automatic prescreening with the confidence of visual examination of sorted and concentrated abnormal cells.

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DR. KAMENTSKY

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The screening of cytologic material is often done by hierarchy of two or more levels of screening. The prescreening philosophy employed by cytotechnicians and pathologists for later verification at higher screening level can also be employed by a hierarchy of different instruments with different screening discriminations and processing rates, or the instruments may prescreen directly for the cytotechnician or pathologist. This prescreening can be performed on a sample basis, whereby the instrument indicates whether given sample should be considered by

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sec) In this way each cell in the suspension is
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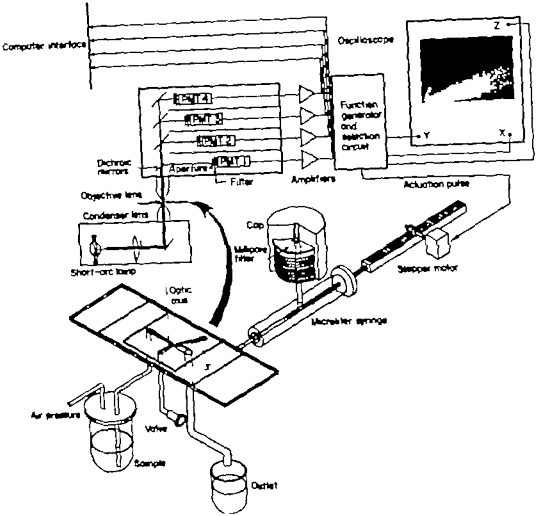


FIG 2 Block diagram of the cell spectrophotometer and sorter

necessary to ensure that the desired cell had been captured in the side channel Thus, several other cells will accompany the selected cell We were successful in achieving a final concentration of selected cells of about 1 5 from initial concentrations in the range of 1 10,000 The selected cells were drawn far enough into the side channel to remain there for the duration of run

After sample had been processed the main channel was automatically flushed in reverse for leaning A membrane (Millipore) filter was placed on holder connected by tubing to radial hole drilled into the barrel of the syringe as shown in Figure 2 During the wash the plunger of the syringe was withdrawn to just beyond the radial hole so that there was flow of clean water through the side channel, the syringe, and the membrane filter The separated cells were thus flushed out of the side channel and trapped on the filter The filter was then removed, placed on a slide and stained for inal observation of the cells The cells are all visible in one low-powered microscopic field as illustrated in Figure 3

There is no reason why the cells need be collected on filter The separated fluid could be collected in syringe for experiments, for example to separate out specific live cells to start cultures The collection line could flow to another instrument for slow more detailed, measurements of the smaller number of selected cells

I have described tw different techniques for sorting biologic cells using fluid transport system measuring optical or electrical properties as basis for sorting There are large number of cytologist. In this case the instrument should not miss more positive cases than the cytologist and must screen out a sufficient percentage of negative cases to increase the overall efficiency of the laboratory at a reasonable instrument cost.

As a second alternative the instrument can screen on a cell basis and either mark or separate off suspicious cells for subsequent consideration by a cytotechnician or perhaps by a slower but more discriminating instrument. For potential prescreening instruments using slides to transport cells, the position of suspicious cells could be correlated with the stage position by recording the coordinates on some storage medium such as computer magnetic or paper tape. It is conceivable that the magnetic material could be attached to the slide In a tape transport system this marking is simpler since the cells are strung out linearly and the tape can be punched or magnetically marked at the same linear position as suspect cells

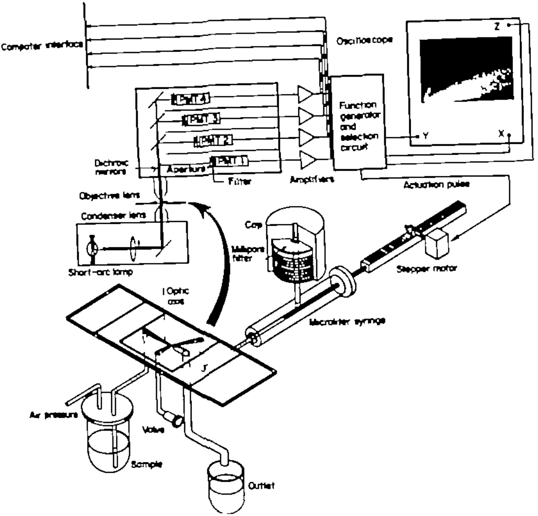
The use of a fluid transport system for cells provides the unique capability at present to physically sort or separate out cells based on some instrumental criterion Two different physical principles have been reported to physically separate out specific cells.

Fulwyler published a report in 1965[1] in which he described a particle separator using a fluid transport system. The Coulter principle[4] for measuring cell size was ingeniously coupled to a chart recording technique developed by Sweet[5] to produce an instrument for sorting cells based on Coulter volume measurements Cells in suspension were passed through a Coulter orifice The liquid stream after leaving the volume sensing orifice into air was broken into droplets by a mechanical vibrator at the stream outlet The falling droplets were small enough so that on the average one of seven contained a cell A voltage pulse timed properly and proportional to the sensed volume was connected to a ring through which each droplet fell to charge it proportional to the volume of its contained cell A pair of fixed voltage deflection plates placed below the ring deflected the droplets The net effect of this arrangement is to deflect each cell containing droplet by a distance proportional to the volume of the contained cell The droplets were collected in containers so that cells within a given volume range were separated from the remaining cells

Although this technique has been reported to separate cells based on volume measurements, there is no fundamental reason why other parameters in addition to volume cannot be combined to establish a criterion for separation using the Fulwyler or other cell sorting techniques I would like to describe a cell sorting system devised by Melamed and myself that was developed to separate cells based on multiple optical measurements. Beginning in 1964 we studied several different separation systems also based on Sweet's work, using droplet formation and electrostatic deflection. They were not adopted by our group because such systems were found to restrict the optical design of a measurement system too severely A separation system based on fluid switching principles popular about 1964 for computer design was developed so that a cell sorting system could be contained on a quartz slide This system is described next.

Figure 2 shows both the optical and mechanical arrangements in the cell spectrophotometer and selector The basic principles for measuring photometric properties have already been described in this symposium. The system we used allowed for simultaneous measurement of absorption, scatter or fluorescence at four different wavelengths in the ultraviolet and visible regions by the use of a series of dichroic mirrors to divert successively longer wavelengths to four photomultiplier tubes We have used various combinations of wavelengths and bright and dark field optics selected to study unstained or stained cells. The photomultiplier signals were processed to obtain specific functions of the measurements. A selection circuit provided an activating signal whenever a specific cell exhibited photometric properties within a predetermined range. For example nucleic acid content of each cell was estimated by total absorption at 2537 Å, and cellular size was estimated by visible scatter Whenever the ratio of absorption to scatter exceeded a specific value an actuation pulse was produced The actuation pulse initiated a cell separation as described below

The cells suspended in a fluid medium were pumped by pressurization of the sample container at a flow rate of 0.5 ml min through a flow channel shaped as shown in Figure The cells were observed at the optical axis by the spectrophotometer and flowed to the sorter junction in 2 msec Unselected cells continued to flow out into the outlet container If a specific cell had optical properties causing an actuation signal, the stepper motor was pulsed, causing the plunger in the syringe to draw 0.03 μl of fluid This fluid pulse was propagated across the flow channel at the junction, causing fluid flow across the junction for about 3 msec There was a delay of 2 msec from the time of detection until an appreciable flow occurred across the channel matching the delay time of the cell to reach the junction from the observation point. In practice the cells flowed at varying velocities, because of differences in position across the channel. To account for this the actuation time was made longer than



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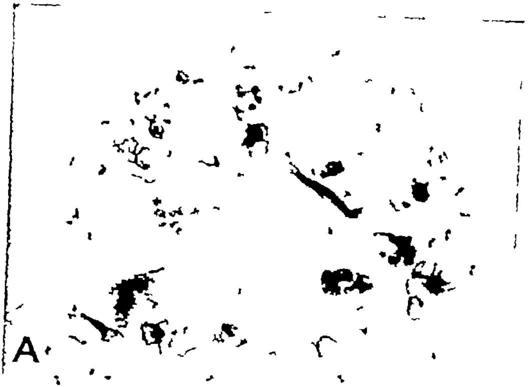

FIG. 3A

FIG. 3B

FIG. 3. Cells with high ultraviolet absorption selected from vaginal wash specimens of patients with carcinoma of the uterine cervix. The membrane filters were stained with hematoxylin and eosin. A. Photograph of the complete area of the filter containing cells (×140). B. Higher powered (×330) photograph of A. C. Section of filter of another case at higher power (×550).

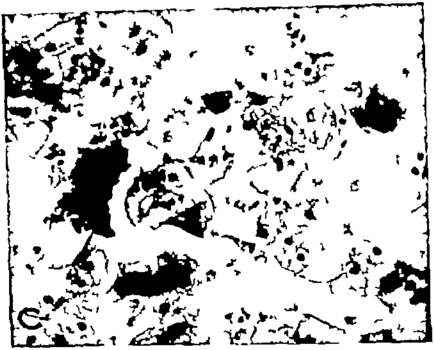

FIG. 50

ariations of both the Fulwyler technique and our own. The system w developed will require considerable engineering before it could be useful in the laboratory unattended by physicist. Such engineering will be intensively pursued during the next few years.

I closing I would like to indicate that the cell sample could be preprocessed before sorting, even further extending the philosophy of processing hierarchies. Such techniques as density gradient separation, electrophoretic separation, selective lysis, selective filtering by column or mesh, centrifugation, or settling are potentially useful for preprocessing samples before introduction into more discriminating instrument for prescreening.

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MR. MEYER

Marking or Sorting of Prescreened Cells with Special Consideration of Marked Cell Tracks

Introduction

On investigating the manual slide method, it was found that it did not lend itself readily to semi- or fully-automatic screening. Mucus helps the cells adhere to glass slide and smearing causes the cells to be laid down in streaks which are patterned by the position in which the material has been removed from the cervix by the spatula. Thus, sampling under these conditions is not random in that any unit area of the slide is not equivalent to any other unit area. As result, very large area of the slide must be sampled to maximize the reliability of finding suspicious material.

The requirements for semi- or fully-automated screening system are that well-populated suspension of the cellular material should be obtained and it should be laid down in random distribution, so that minimum of the sample may be scanned with maximum reliability of detection, as any unit area will then be equivalent to every other unit area. A further requirement is that an area containing suspicious cell can be marked in such way that this area can be easily recalled. The method which lends itself to instrumentation is first to draw a mono-cellular layer of the suspended material in a line *so that the breadth of* the line can be thought of as negligible and therefore a cell within the line can be located easily by using one co-ordinate only The simplest form of marking is to punch a hole because such a mark is permanent and requires only a simple detection system. To obtain an area of cells equivalent to that of the standard glass slide requires a line of approximately 650 mm. in length by 1 mm. width The material best suited to replace glass is one which has the same optical properties and the same inertness to chemicals, and which can be punched without difficulty

In the semi automatic screening system applied to date a two-parameter measurement has been used. This measurement screens out the bulk of the normal cells, and the variation of the *pathologic cells from the normal, in having these* two parameters ensures it is detected and marked. The marked areas are viewed by a cyto-technician for further classification Therefore, the principle of this simple scanning system is to eliminate a greater part of the searching through specimens for abnormal cells.[9, 10, 11]

Sample Collection and Preparation

The cervix is sampled by scraping with an Ayre spatula The contaminated end of the spatula is immediately placed into a fixative which contains a mucus solvent. This fixative has been so designed that the cells are fixed and preserved at the same time as the mucus is dissolved off *the spatula*, leaving the spatula clean of cellular material and the material itself in a fairly fine suspension there are no clumps caused by the mucus left, only naturally occurring aggregates of cells. The suspension is concentrated by allowing the cells to sediment into the cone in the base of the fixative vial and the excess fixative is drawn off by means of an aspirator attached to the graphing machine.

The Graphing Machine

A specially prepared optically transparent plastic tape is used to replace the conventional glass slide the coating on the tape causes the cellular material to adhere to the surface while drying and prevents its being washed off during the staining process. The concentrated suspension of cells is well shaken and drawn up into the pen unit, which is a linear reciprocating peristaltic pump through which is threaded disposable silicon rubber tubing A number is attached to the tape and a duplicate to the patient's record sheet. The plastic mounting tape then passes beneath the pen from which is expressed the cellular material, and this dries into a line 1 mm. wide by 650 mm long A fresh piece of tubing is pulled through the pen for the next patient's specimen The specimens are usually batched in 50's onto a spool ready for staining

The graphing machine is shown in *Figure 4*

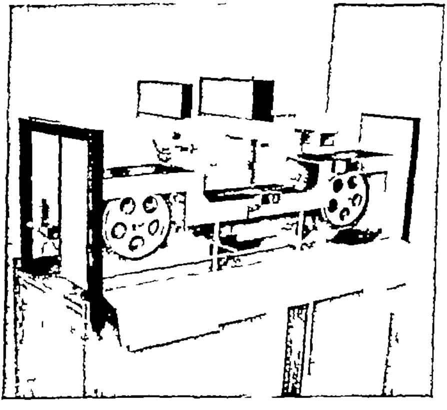

Fig. 4 The graphing machine

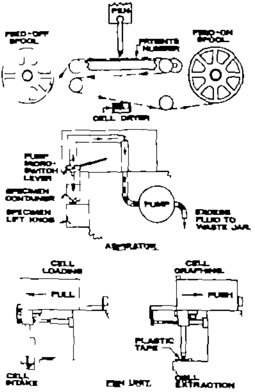

FIG. 5 General mechanics of the graphing machine

and the general mechanics in the schematic diagram, Figure 5

The Staining Machine

The spool of specimens is stained i continuous manner using the Papanicolaou technique After the specimen leaves the last xylene bath the line of cells is covered by a mounting material having the refractive index of glass The width of the mountant track is approximately one and one half times that of the sample line ensuring that all material is adequately covered The standard coverglass of the slide is replaced on the tape by tri-acetate adhesive film The time taken to stain 50 specimens is 62 minutes

The staining machine is shown in Figure 6 and the general mechanics are shown in the schematic diagram, Figure 7

The Viewing Machine

The spool of stained tape is mounted onto multi-functional tape transport system built round conventional microscope The functions are automatically performed and they are

(1) Fast L – R and R — L to enable any pa ticular specimen t be found rapidly along the length of the tape

(2) A continuously variable mode for manual iewing of any particular specimen and for the manual punching of fields of interest for reference, or marking for second opinion

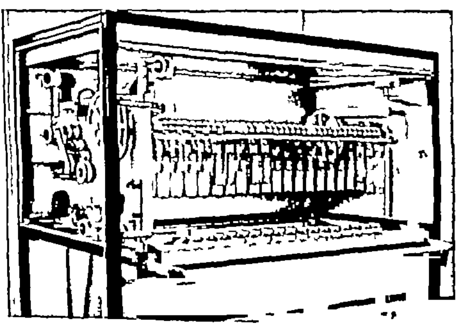

FIG. 6. The staining machine.

(3) A constant speed auto-punching mode during which detected large nuclei are marked by means of punching a hole in the tape.

(4) A playback mode in which the reference holes are picked up in such a way that the area of interest may be viewed by the operator from the microscope objective field.

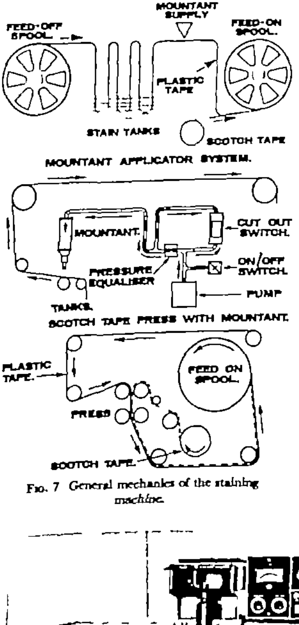

Fig. 7 General mechanics of the staining machine.

The viewing machine is shown in Figure 8 and the schematic diagram is shown in Figure 9

Scanning Unit

An image of the object plane is projected onto a screen by means of a mirror situated above the ocular of the microscope. The mirror is one of 18 mounted on a drum. When the mirror drum rotates, the image of the object plane is scanned across one diameter As the specimen moves at right angles to the direction of rotation of the mirror drum, a two-dimensional raster is produced The size of the rastering spot is given by

$$\text{Size of spot at object plane} = \frac{\text{Diameter of circular aperture in image plane}}{\text{Magnification of object in image plane}}$$

Behind the aperture is a semi-circular holder in which a series of neutral density filters are mounted with one slot left empty to give an unobstructed light path. Any filter can be selected to intercept the light path from the image plane aperture The mechanics of the scanning unit are shown in Figure 10

Classification of Intercepts

The projected image of a cell passing over the aperture will change the intensity of the light transmitted through the aperture. This change is detected by a photomultiplier which will produce a voltage pulse of width equal to the time taken *for the cell to cross the aperture and of amplitude*

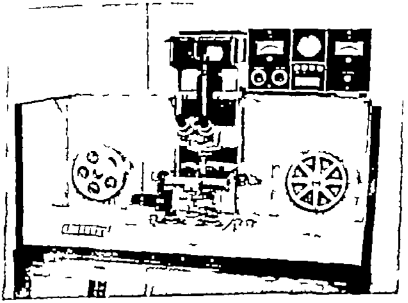

Fig 8. The viewing machine

equivalent to the density of the cell. As the stain taken up by the nucleus gives it density greater than that of the surrounding cytoplasm, the maximum amplitude of the pulse is created by the nucleus When the pulse from the nucleus reaches preset amplitude it generates a second pulse of width dependent upon the time that the pulse remains above the preset point T select specific group of intercepts to be recorded, when the primary pulse passes the preset amplitude a gate is closed for period equal to the minimum intercept length required to be measured. The gate is then open for period such that the sum of the minimum intercept length and the length the gate is opened are equal to the maximum intercept length tha is to be recorded If the secondary pulse width is smaller than the minimum intercept length set, it cannot be passed to the recording stage as the gate is closed Only secondary pulses which decay during the period the gate is open can be recorded This is show in Figure 11

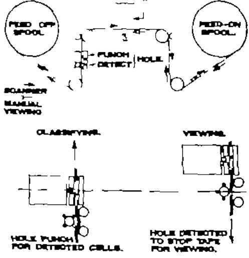

FIG. 9. Schematic diagram of the viewing machine

As the amplitude discriminator is fixed, the nuclei to be selected must have density above a determined level. The amplitude is adjusted by expanding the pulse between the baseline and the fully obscured scanning aperture for given density by means of selected neutral density filter This is achieved by setting the baseline at zero, intercepting the light path through the aperture with the filter and expanding the resulting signal to a predetermined limit The filter is then removed and when nucleus intercepts the aperture and completely obscures it If its density is less than 90 per cent of the density of the filter it will not pass the selecting threshold. Therefore, only nuclei which ha intercepts of size that can pass through the gate and are of density which ca pass the amplitude selector are recorded

The number of tercepts recorded depends upon the size of the nuclei, on the umber of nuclei present and on the number of scans per unit length of tape, assuming that the length of the scan covers the sample width The general theory of scanning of objects randomly distributed in plane is due to number of authors whose publications cover some 40 years— Adler Cournbee Dewes, Hawkesley Taylor[11] and Wicksell The theory is based upon obtaining a size distribution of particles randomly distributed in plane by measuring chords The number of i tercepts for distribution of particles whose size range is between and D with the cut-off at (+ G) is given by the integral equation

$$\phi(I) = \frac{S}{H} \int^{D} N_D\{(D^2 - {}^2)^{1/2} - [D^2 - (+ g)^2]^{1/2}\}\, dD \qquad (1)$$

Where S = Number of scans
H = Track length

For simplicity N is the same for all values of D and N is the number of particles in a particular size range

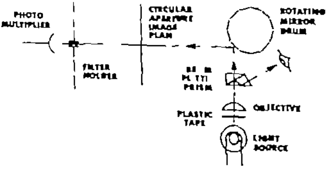

FIG. 10 The mechanics of the scanning unit.

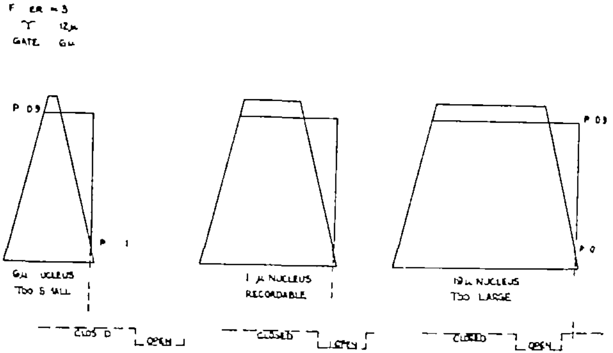

FIG 11 Schematic diagram of recording of secondary pulses which decay during the period the gate is open.

The probability of obtaining at least one intercept on a nucleus whose size is between τ and $(\tau + G)$ is given by Poisson's formula for rare events, which is

$$q = 1 - e^{-N\frac{S}{H}\sqrt{\overline{D}^2 - \tau^2}} \qquad (2)$$

Where $\overline{D}$ = mean diameter of nuclei over size τ

Setting Parameters

Diamond carried out measurements to ascertain a method of selecting suspicious cervical material from normal. His work showed that specimens could be classified into suspicious or normal by grading them as to whether they contained many nuclei greater than 12μ in both minimum and maximum chords or not. His publications,[6, 7] have shown that the method of selecting slides having nuclei greater than 12μ by an unskilled operator has a reliability of not missing any suspicious material, and less than 5 per cent of negative material is incorrectly classified This latter include post-menopausal, trichomonas infection and post natal cases

Because of clumping of cells, nuclei of small cells can overlap and present chords of over 12μ therefore an upper intercept cut-off is used The instrument has been set to have a cut-off such that objects greater than 20μ will have a very low probability of producing punches, but there will be a maximum probability of detecting between 12μ and 20μ

The sample laid out on tape was to present a similar population to that obtained on a well-made glass slide smear[3] The method of staining was that of Papanicolaou the reason for this is that the final diagnosis is made by a cytotechnician who has been trained in reading this type of stained specimen The automatic staining procedure allows for a consistency of staining because of the constant time the specimens remain in the solutions, and the stains are replaced after every 400 specimens Any variation in *staining* can be allowed for by using a filter of density equivalent to that of the nuclei of interest, to be interposed between the scanning spot and the photomultiplier The range of the filters is from extinctions of 0 8 to 0 4 in steps of 0 1

Where there are high concentrations of large nuclei the marking of the specimen will become excessive As it is the intention that the instrument will alert the cytotechnician to suspicious specimens and will not give a detailed identification of all the suspicious material within the specimen, a restriction on the number of fields that can be identified has been built in This takes the form of a deadlength once a field has been marked, the system is inactivated for a length of 10 mm It has been calculated that this does not in any way affect the probability of detecting large nuclei in small numbers in any specimen and this has been confirmed by experimental tests

After the instrument has automatically scanned the 30 specimens batched on a reel the technician plays the specimens back and the tape automatically stops where a hole has been punched and the associated field is visually observed. If the field shows suspicious material the operator can scan the specimen manually and mark any other field which will uniquely identify the class of specimen. If, on the other hand, the fields show only inflammatory changes or large nuclei of benign origin, the operator by depressing key starts the tape running until it is stopped by the next punch hole. This method, therefore, allows the cytotechnician to utilize his training to the full but reduces long tedious searching of negative material

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Dr. Tolles

Specific Consideration of the Operational Characteristics of Cell Marking, Cell Selection, and Flow Through Systems

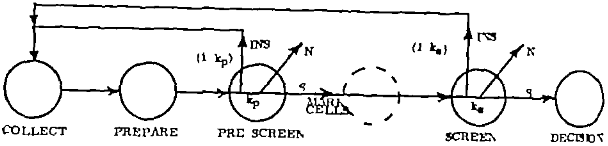
Cell Marking Systems

The upper diagram of Figure 12 depicts the functional flow diagram of a cell marking system. The specimen is accessioned at the left side of the figure prepared for deposition on transparent tape and stained in highly mechanized manner The prescreener through its high-resolution scanner classifies each absorbing event and makes mark on the tape opposite those events judged to be suspicious After number of specimens have been so processed, the tape is forwarded to the screening technologist A scanning device associated with the viewing microscope automatically locates those events judged to be suspicious by the prescreener and the technologist makes his judgment of these marked events

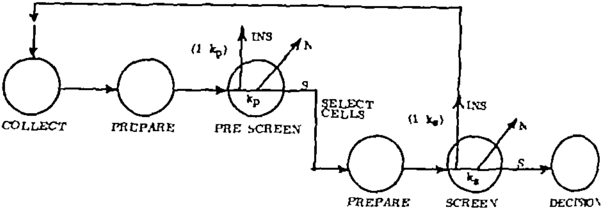
One of the attractive properties of this system is that the human screener is asked to look at the identical cells which the instrument identified as suspicious, not at companion aliquot of the original specimen Another attractive feature is that there is no need for second staining procedure as the one chosen is acceptable to both machine and man

As no reports of clinical trials of this type of system have appeared, it is pertinent to ask some questions about its operation at this time As indicated in Chapter VI, the first operational characteristic to inquire about is the throughput factor k_p Has the preparation of the specimen for machine reading reduced the abundance of cells, or selectively diverted certain classes of cells, in such way as to produce a low value of k_p?

Next, the accuracy attainable is of fundamental interest, and its relationship to the throughput factor should be known. In this case, there may be special penalty attached to in the occurrence of high value of false positive indications inasmuch as each such event is brought to the technologist' attention Will the technologist' throughput k_s be influenced by the prescreener? Finally the developers of this type of system will be required to show in lengthy and carefully designed clinical test that the system as such is technically adequate and that its inclusion in the screening loop improved the delivery of health service over the unassisted routine screening system



FLOW DIAGRAM OF SLIDE OR TRACK MARKER



FLOW DIAGRAM OF CELL SELECTOR

Fig 12.

Cell Selection Systems

This type of system is diagrammed in the lower drawing of Figure 12.

The cellular specimen is collected, prepared for the flow-through optical system and submitted to a low-resolution, bulk-property detector which *may measure as many as three or four separate* physical or cytochemical entities of each particle as it presents itself Criteria of suspicion are applied virtually instantaneously and those cells which qualify as abnormal are diverted from the *analytical* current *into a collecting* vessel. *The* downward displacement of the diagram indicates a second line of cell preparation and manipulation. At the end of the run, the suspicious cells (along with some obligate normal cells) are concentrated and stained *for* observation *by* the technologist.

The low optical resolution is attractive because of the attendent increase of speed of proceeding in comparison with systems depending on micromorphology for the identification of abnormal elements.

The throughput factor for this system has already been shown to be low and its improvement is crucial to its ultimate success

e selection of abnormal cells results in a concentrated specimen which should make the task of the human screener much lighter However the throughput factor at the technologist (k_s) must be considered. Finally the cost effectiveness of the prescreener versus the unaided *system must be evaluated.*

Flow-through Systems

The function diagram of a flow-through system is similar to the cell-selector diagram. *If the* prescreener has judged the specimen as a whole to be abnormal, the specimen for subsequent decision may be *obtained* from the cells flowed through the prescreener concentrated and stained for human inspection. Alternatively the specimen for human review may be obtained as an aliquot of the original specimen.

As in the previous cases, *evaluation of the* operation of this type of instrument requires the determination of k_p, FN FP k_s and the cost per specimen so processed

This type of system is transient in nature. An optical event occurs, and is classified and *enumer*ated, but the *cause* of that event has passed and cannot be recaptured, examined or rationalized. This presents a serious problem of evaluation of operation and control of the instrument. In the long range, the simplicity *of this* proposed *system* is very inviting but in the short range it poses many problems of control and understanding of its individual responses

Discussion

DR. HEALEY

There are four papers offered for discussion under the title of Sorting or Marking the Prescreened Cells Two are concerned with cell suspension flow systems, one with cell tracks on film, and one is of more general nature.

Fulwyler describes method for separating drops of liquid containing cells into separate containers The cells are first sized by being measured by the Coulter principle He states that it is possible to make several serial measurements before the cell passes through the sorter and in this way multiparameter characterization of cells could be achieved It would seem that this would require careful correlation between signals and cells He further states it would be reasonable to sort six classes of cell. With more than six classes one expects that some would have to be lumped together and reprocessed

Kamentsky describes fluidic method of abstracting cells of interest at right angles to the main flow of cells being measured The cell of interest is usually accompanied by number of neighboring cells unlike Fulwyler' method where the cell is usually alone. Kamentsky further describes the preparation of such sorted cells for viewing by cytotechnicians Both these systems would appear to give adequate sorting and implicitly rely on further analyses for a final diagnostic decision after transforming the environment of the cell

Meyer describes method which imitates, as far as possible, the traditional Papanicolaou stained smear using film rather than slides The criterion for marking suspicious cells is the recognition of nuclei with minimum and maximum *cords greater than 12 microns Marking is per*formed by punching the film alongside the selected cells. Mechanical recall then enables cytotechnicians to view marked cells Given that the 12 micron criteria is adequate to eliminate false negatives the method would appear to stand or fall on the ability of the one parameter device not to mark too many false positives As a whole, it is an example of one parameter prescreening system, but the method of laying cells in narrow track and marking the film to the side of the cells as more general application.

Tolles reviews the methods mentioned above and is interested in their accuracy He states that their cost effectiveness is crucial and argues that the flow systems because of the change in mid stream may give rise to serious problems of operation and matching

The methods described and variations and improvements of them would seem to offer adequate sorting and marking of cells. However the crux of the matter is what is marked and with it the feasibility of prescreening, which topic is outside the present discussion

DR. HUSAIN

The consideration by Tolles of the three systems—cell marking cell selection and flow-through techniques, presents preference for the first system It is the one most favored and pursued in England and perhaps the easiest to create and control It is one in which development of various pattern recognition scanners, both the simple seeker and the more complex analyzer forms has the highest impetus

It is here that the simple detection and identification by whatever criteria or parameter by which the scanner is programmed to detect will be immediately evident and recognized by eye. The Vickers scanner is at present on such trial, with as yet unproved effect The new Quantimet Image Analysing Computer Scanner (The Cervical P Q M) will in our hands, we hope, go long way towards the evaluation of such track laying and parameter selection systems. That the technologists through-put (k_e) will be influenced by the prescreener is inevitable From our calculations, it is likely that a machine will easily read 45,000 to 50,000 cell samples per year and this would be controlled and checked by one screener probably not working full time, i about 5 to 10 times the normal rate of output

The accuracy and false positivity will be the key values by which each technique will be judged. The cost of the average instrument will be of the order of 5 to 10 times the annual salary of cytotechnician, and as it is expected that instrument-depreciation and running costs will occur about the rate of one-fifth to one-tenth of the capital cost per year this should provide a convenient way of equating the cost of such automation

The cell selection and flow-through systems are ones which work on the simplest parameters and therefore do form of mass sorting which results in concentration of the suspect cells. This also is acceptable so long as the "miss rate" is kept low and the true positives are not buried in too great false positive group. The evidence being evanescent except in such side shunt systems as in the Kamentsky spectrophotometric apparatus, there is much greater need for accuracy in final identification than for the cell marking techniques.

The technique of rapid cell analysis in fluid transport on the simple parameter of nucleo/cytoplasmic ratio by Kamentsky has been developing over the past three years and now results in the presentation of the suspicious cells separated onto membrane filters.

Both Fulwyler's and Kamentsky's techniques of cell sorting by fluidics present intriguing possibilities both of speed and convenience as the deposition for tracking or final identification by eye or analytic pattern recognition can be made a continuous process.

The technique by Fulwyler probably will disrupt cell clusters for eventual pattern recognition while the Kamentsky technique will probably not separate the cells enough without a brisk dispersing or disrupting technique

With regard to Fulwyler's novel electronic separator one needs to ask a few questions As most of his work has been conducted using blood cell one poses the problems we face in the field of exfoliative cytology Can this technique work on a mixture of epithelial and mesodermal cells mixed up with mucus and serum and bacteria and the like?

How would he prepare such specimens with cells in a separated form, that is to destroy the inter-cell boundaries and connections? Would any enzyme or chemical stripping of the surface mucopolysaccharide or mucoprotein affect the ability of the cells to take a charge on their surfaces?

His technique is certainly speedy and we will watch its development with interest, especially whether the dysplastic and malignant cells are separable from the rest, as opposed to simple separation into cell genera

The contribution of Meyer is of fundamental value in the approach of partial automation providing selected fields to be studied by eye

His hypothesis, however is based on certain assumptions not necessarily fully proved as practicable or even true In this he is not alone

The existence of some large nuclei over 12 μ in most malignant smears is probably true There are however a few small carcinoma in-situ and some glandular cell tumors in which the nuclei may well be below 12 μ in size Moreover there is the matter of cell clusters where any scanning machine of relative simplicity will have to "gate out" or cancel cell clusters, as they would otherwise include many benign endocervical and scraped squamous cell fragments

There is also the fact that measurement by chord length alone is an insufficient criterion for detection, as many large and degenerate and hydropic nuclei, especially from the endocervical cells will be signalled The detection, as Mr Meyer has implied is one based on a particular eshold (of light absorbence) Scanning exclude the faintly-staining and de leus

Our work with the Quantimet Image Analysing Computer using a single spot scan with line memory and potentiometer circuits for both horizontal chord size and density (light absorbence) has demonstrated two vital things Firstly that only proportion of malignant nuclei of any size ar accessible to automatic scanning by virtue o being separate and not buried in a cluster Some times these separate signals are quite few i number or too degenerate or too small to be de tected Secondly that there appears to be an in verse relationship of density to size of malignan nuclei where the smaller nucleus is usuall darker while the largest malignant nuclei may b considerably more faintly or lightly stained. S the threshold density band between malignan *and benign cells can be drawn at a tangent There* appears to be a lower limit of 10 μ below which polymorph nuclei (of 8 μ) and superficial squam nuclei (of 6 μ) will confuse the picture

The infrequent positive signals may also be lost if an instrument depends on a negative phase following a "positive" signal being punched to prevent the tape being shredded This occurs on the Vickers machine where a negative phase of 1 cm occurs It therefore follows that a more refined form of sensor should be used than a simple slit scanner to build up an image and also that a form of marking a signal on the tape commensurate with its extent should be used. Electromagnetic or some optico-electronic technique as is being developed by the Tetronic cytotrack, is the better method of signalling for recall The new Quantimet does provide a fairly accurate and detailed build-up of particle size form and outline and this should permit a reduction in size of malignant nucleus detectable to around 10 μ. The maximum use of nuclear margin and associated cytoplasmic density readings can be made at an economic degree of resolution using a television spot scan, and these will help to exclude more definitely the irregular-edged neutrophil and abrupt-edged artefact

Meyer has perhaps not sufficiently stressed the need for suitably-surfaced tape, perhaps because his Neccoloidursed Melinex tape from Imperial Chemical Industries has been the most successful so far encountered by u The surface is adequately ater ettable and the Melinex strip stands any amount of processing through the usual staining and dehydrating solution

The covering of the mountant by triacetate adhesive film (Scotch tape) is a novel idea and well performed but suffers from producing the entrapment of air at times and of "ribbing" if the tape be rolled at too acute an angle Both produce a degree of background noise.

Here the use of a suitable acrylic resin might prove of greater success as seen in the cytotrack method.

We have discussed cell preparation techniques and stain schedules elsewhere. These are paramount prerequisites for satisfactory cell marking.

DR. MELAMED

It is clear that some effective automated method to identify and separate cells with predetermined properties would be very valuable. Studies of cell identity could be simplified, particularly in correlating various modalities of instrumental measurement with traditional morphologic classification. Specific cell types could be accumulated in relatively pure populations for biologic experiments.

Three different systems of cell marking or cell sorting have been proposed in the "Opening Remarks." Two are designed for fluid transport system of cells in suspension, the third for system that transports cells placed on tape. Each has certain advantages and disadvantages, and each has been used to identify or separate cells as part of total system of automated cell measurement. To some extent comparison of these different methods of cell marking or cell separation is influenced by other aspects of the systems in which they have been used. Nevertheless, it is useful exercise to compare these methods in judging the current state of the art (Table I).

While all three methods of cell marking or separation function very well for specific purposes, none is suitable for all. The initial hurdle has been cleared in showing that these devices can be built at all. During the next few years we should see them considerably improved, and perhaps augmented by instruments based upon still other principles.

DR. SPRIGGS

First clear distinction must be made between the principles of *sorting* and *marking*. *Sorting* I take to mean the separation of specimens into two sets, positive and negative. In prescreening system, positive smear would be one requiring to be examined by human, and negative would be passed as normal. *Marking* is the automatic spotting of certain cells for human viewing, so that only selected fields need to be examined by the human observer before passing smear as negative.

Sorting

In every sorting system so far suggested the decision has depended on *count* of particles exceeding certain criterion. In cervical smear the number of abnormal cells gives an idea of the size of the lesion sampled by the spatula, but the diagnosis does not (or should not) depend on this, but rather on the characteristics of the cells themselves. In some "positive" smears the malignant cells are greatly outnumbered by normal cells, so that they have no influence on the *mean* value of any parameter and may not even form very substantial secondary population. Moreover, in almost any conceivable sorting system, false negatives (the most serious error) could only be reduced to acceptable levels at the cost of large numbers of false positives. Unless the system used included the making of conventional smear at the same time, the calling back of substantial number of women for repeat smear would make it unacceptable—a point clearly made by Wied.

These defects of a sorting system make the alternative, *marking* system, very attractive.

Marking

The principle of this system is to mark the cells which have been signalled according to certain criteria, so that these fields can be played back to the human observer. The prototype is the Vickers machine described by Meyer in this Symposium.

Let us assume that very simple criterion has been chosen, and that any field is marked containing a particle stained to a certain density with hematoxylin and with vertical diameter between 12 and 20 μ. Positive smears from squamous carcinoma-in-situ and dysplasia virtually always contain nuclei in this range, as anyone can verify. (There is no intention to mark every malignant cell, most of which have nuclear diameters within the range of endocervical cell nuclei; the parameter is chosen such that every smear which we wish to report as positive should have at least one field containing recognizable malignant cell marked by the device.) It is likely that most of the objects marked will be artefacts, particularly rolled or overlapping cytoplasm, and negative smears will be marked as well as positive ones. The human observer looks at marked fields. If all the marked fields are passed as containing nothing suspicious of malignancy, the whole smear is negative. If a cell is seen which raises any suspicion, the observer examines the whole of the smear in the usual way and reports what he sees. No counting is done, so no cut-off between positive and negative needs to be decided upon.

A great advantage of this system is that it can make use of parameters which characterize only a very small proportion of the cells present. We already know that there is no absolute criterion of malignant cell, but there are criteria by which we can pick out *some* malignant cells with high reliability. Another advantage is that there is continuous human monitoring of the quality of the smears. A third, and even greater advantage, is that it is extremely simple compared with most of the multiparameter or pattern-recognition systems proposed so far

The last statement invites the question, "If it is so simple, why has it not yet succeeded?" The answer of course, is that the familiar technical problems have not yet been solved these range from the problem of identifying a particle of a certain size by measuring random chords, to the problem of staining nuclei without at the same time staining cytoplasm But these difficulties are not reasons for throwing out the principle if we cannot achieve simple vertical measurements of nuclear diameter how can we hope to do the more sophisticated measurements such as nucleo/cytoplasmic ratio, estimates of nuclear irregularity and so on? In my opinion the simple marking of large nuclei is the minimum automated cell screening method which must be achieved before we consider anything more complex or more efficient, and I should like to see many centers attempting this, rather than judge the system by the performance of a single machine To aim in the first place at a completely automated sorter of smears is like attempting a moon landing before trying earth orbits.

Assessment of Sorting and Marking Machines

If a sorting machine is claimed to be effective it should be easy to test it by comparing its performance with that of a cytotechnologist. Its mistakes can be classified as false positives and false negatives, and these will be the important figures to obtain in any field trial.

If a marking machine is subjected to a field trial of this kind, the results can only be very misleading False positives are impossible, because the machine does not specify which smears are positive, and the decision rests with the observer False negatives certainly can occur due to failure of the instrument to mark any field containing

TABLE I

RELATIVE ADVANTAGES OF CELL

	Physical basis of cell marking or separation	Number of modalities measured	Sample preparation	Fixing and staining	No. cells examined in each specimen	Rate of cell examination
Tape transport	Mechanical	One (nuclear diameter in one axis)	Moderately complex	Required	Uncertain (not all)	Moderate
Fluid transport charged droplet deflection	Electromagnetic	One (cell size or cell fluorescence)	Simpl	Optional (non for cell sizing)	All	Ultra-rapid (2000 cells sec)
Fluid transport Fluid switching	Mechanical fluid switching	Two to four (nucleo proteins, cell mass, total proteins, cell viability or other pe cifi staining p operties)	Simple	Optional (depends on modality to be measured)	All	Ultra-rapid (1000 cells/sec)

malignant cells in what should be positive smear But in the great majority of smears from patients with carcinoma or carcinoma-in-situ there are malignant cells in nearly every field, so that even a *random* marking of smears would result in the correct report being given. The best method of discovering the reliability of the machine would be first, a test in which the machine is challenged with selected smears containing small numbers of malignant cells only if this test is successfully passed, an assessment of the false negative rate in screening conditions will be worth doing and finally the appropriate working trial should assess the advantage, if any achieved by the machine in saving of time and money this, after all, is the foreseeable object of automatic scanning any improvement over the human performance being much more remote aim

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DR V DELLA AND MR T TRUJILLO

These four papers have one common theme (i e how to make available for visual examination by the cytotechnician or cytologist the cells considered as suspect by the prescreening machine) Three of the papers (Fulwyler Kamentsky and Meyer) deal with actual equipment already designed and functioning to various extents along these lines The fourth paper (Tolles) briefly considers three possible approaches and some of their merits and disadvantages Of the three papers describing operating equipment, two deal with the flow transport-cell sorting method (Fulwyler and Kamentsky) and the other with tape transport (Meyer)

Flow Transport-Cell Sorting Systems

This system offers the attractive potential of discarding very large numbers of normal, uninteresting cells and concentrating the suspicious cells in a convenient way for subsequent decision-making by human examination (or by machine when available) For example, with the Fulwyler

SORTING OR MARKING DEVICES

Specificity in sorting or marking predetermined cell types	Selectivity in sorting or marking predetermined cell types	Interference by cell clustering	Correlation of instrument signal and light microscopy morphology	Cell separation for biological experiments	Cell separation for biochemical and physical experiments	Specimen preservation for re-examination
Fair	Fair to good (depends on cell concentration)	Yes	Easily done for individual cells	Not possible	Handicapped because cells are fixed and stained	Yes
Good	Very good	Clusters interfere in cell sorting	Compares groups of cells in like classes difficult for individual cells	Easily done	Easily done	Possible (unfixed cell will change with time)
Good	Fair to good (depends on cell concentration)	Depends on size of cluster and modality measured	Compares groups of cells in like classes difficult for individual cells	Easily done	Easily done	Possible (unfixed cells will change with time)

continuous human monitoring of the quality of the smears. A third, and even greater advantage, is that it is *extremely simple* compared with most of the multiparameter or pattern-recognition systems proposed so far.

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volume-measuring cell sorter has been used mostly with blood cells however at the University of Colorado the device has been extensively applied to dispersed mouse spleen cells and bone marrow cells The problems to be encountered with vaginal and cervical samples are much greater as Dr Husain points out, because of the presence of mucus cell clumps which must be dispersed, and bacterial infection We are well aware of these problems To answer his question, we do not know at this point in time if cervical samples can be made suitable for processing by our method However, efforts are underway at the Los Alamos Scientific Laboratory and at the National Cancer Institute to resolve this point

Dr Kamentsky

In response to your request for closing remarks on "Sorting Or Marking The Prescreened Cells there is no basis for making closing remarks and I decline from doing so

Mr Meyer.

Does Dr Husain mean that there are some carcinoma-in-situ and some glandular cell tumors where there are no nuclei greater than 12μ If this is true what is the proportion of these cases to the total population of carcinoma-in-situ and glandular tumor cases?

Further to the problem of hyperchromasia, we know that not all dyskaryotic cells and not all cancer cells are hyperchromatic, but again there will be a proportion having the characteristic of large size and hyperchromasia As we know from Dr Husain description of the Quantimet, this instrument counts areas according to average intercept length and select according to density threshold level In his paper at "The Tenovus Symposium Cardiff 1968, Husain states that density played a significant part in separating malignant from benign How then does Dr Husain consider that the V C S A criterion differs from his own

The ribbing of the tri-acetate adhesive film is not due to the adhesive film but to the mountant If the mountant is put on too thickly and therefore does not dry off sufficiently before the adhesive tape is applied, then "ribbing" will occur and this is easily controlled Bubbles are produced in the mountant and not at the time of application of the adhesive film

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The reason for incorporating a negative phase following a positive phase is to reduce the number of identified fields in the case where there are a large number of nuclei or other objects having the characteristics of size and density which we use as markers Once the instrument has identified a positive field or a number of positive fields, it is redundant for the instrument to continue marking since it is at this stage of the analysis that the cyto-technician takes over

Where the number of cells is small, the effect of this dead period is minimal, e.g. if there were twenty such detectable nuclei on average the loss due to the dead time would be approximately four cell

A pulse must reach a specific amplitude which is proportional to the optical density of the object being measured Any pulse reaching and passing this limit is then measured for the length of time it remains above this specific density The time it remains above this level is proportional to the intercept length of the nucleus If this intercept length is greater than 12μ but less than 14μ it is recorded by a hole being punched in the side of the specimen tape This identifies the field containing the cell, nucleus or object creating the pulse

To scan a specimen the area is fixed and the time taken is 1 minute The effect of high concentration is two-fold

1) Overlapping particles will be ignored or

2) Overlapping conditions can produce artefacts which will mimic a nucleus or cell and so will be recorded

The method is that of a line scan and the probability of intercepting an object The number of scan lines per length of 600 mm is 25,000

The method for laying down the track and the fixative we use does not break up natural cell clumps but it does free cells from the mucus In our experience people adjust very quickly to looking at cells distributed in a random manner other than in a smear and this causes no difficulty in specimen identification and classification

Dr Tolles

It is hoped that continued attention will be given to system analysis of these several generic systems simply because of the confusing evidence in some of the discussions on resolution, efficiency of utilization of scanning time and speed of cellular analysis All high-resolution systems are not slow and all flow systems are not of low resolution as one sometimes reads Rather one must take into account all of the variables of the information content of the analysis system, macroscopic resolution, channel capacity and signal noise ratio of the measuring system and the presentation efficiency of cellular data sorter the suspect cells could be deposited in a line on a moving transparent tape—similar to the method described by Meyer except that only cells considered as suspicious by the machine would be deposited on the tape and the length of the line would be much less. Or all suspicious cells of a patient could be deposited within a small area on an ordinary microscope slide along with a random sample deposited nearby for easy intercomparison. In this way much of the tedious examination of normal material would be done by machines (which excel at repetitive boring operation) and a larger fraction of the time of trained personnel could be devoted to suspicious material. As Tolles rightly points out the machine throughput factor should be as high as possible referring to the Kamentsky sorter he says that the throughput factor for this system has already been shown to be low While this latter undocumented statement may be true for this particular system, it should be noted that we are dealing here with a set of systems that have flow transport and cell sorting in common but can have very different cell sensors And it is the cell sensor and signal processing part of the system that determines throughput

Greater cell concentration is achieved with the Fulwyler sorter because of its faster operation which results in only a few per cent of unwanted cells accompanying the selected cells. In the Kamentsky system there are about four times as many unwanted cells as wanted cells. For many purposes either may be acceptable, but for subsequent high resolution pattern-recognition, decision-making machines, the fewer unwanted cells the better Also the presence of many unwanted cells along with those actually sensed as abnormal makes it difficult to determine which cells were responsible for the machine decision

Both systems have a good deal in common Cell analysis rate is high of the order of 10 per minute a fundamental limitation is the requirement that the chance of two cells in th sensing volume be small A variety of parameters combined in various ways (ratios, preset upper and lower bounds et) can be brought to bear on the decision making to sharpen the resolution Sample preparation must be compatible with the requirement for subsequ t human examination

Tape Transport Marked Cells

The system described by Meyer is the most complete and includes provision for semiautomatic deposition of cells from each patient in a line 0.1 cm wide by 65 cm long on transparent tape followed by staining using the Papanicolaou technique Staining time is about 1 minute per specimen The stained tape is then moved along the stage of a scanning microscope and each cell is examined suspicious ones are marked by punching a hole After scanning 50 specimens batched on a reel, the technician plays back the tape which automatically stops at the suspicious cells for visual examination. At the usually occurring cervical cancer rate most reels should be negative

There are several advantages to this method The only stain used is suitable for both machine and man, prescreening by machine and screening by human are done on the same suspect cells, and the concept of a complete, integrated system has been followed. The entire specimen can be recalled if anything unusual turns up Some important characteristics of the system are not discussed or are not clear Apparently a two-parameter measurement is made but in spite of extensive technical detail about the measurement it is not obvious what is being measured and how signals are combined to yield a decision We gather that nuclear size and optical density (or perhaps cell size) are sensed Neither the machine time required to measure a specimen nor the number of cells analyzed per specimen is given The problem of overlapping cells and number of scans per cell are not discussed. As Tolles points out, no clinical trials have appeared therefore throughput factor accuracy and overall improvement over the conventional method are unknown. The system is engineered around the Papanicolaou staining technique and hence cannot take advantage of cellular properties which may be superior for machine sensing Also, visual examination of widely separated suspicious cells may be difficult for a technician who relies on intercomparison of suspicious cell morphologies In other words, cells arranged single file along a 63 cm line may be ideal for the machine but troublesome for the human

Conclusions

It seems that, in the near future sorting or marking the prescreened cells for subsequent human examination will be a requirement of any automated cytology system As Tolles points out, a flow-through system which does not have this feature is difficult to control and to understand If the future brings more clinical experience and sophisticated methods, the sorted or marked cells may be examined by a lower high-resolution machine if prescreening eventually becomes sufficiently reliable this whole function may be eliminated For the short range feel that sorting or marking is necessary and that, although both methods need proving out the sorting approach holds the greater potential

Closing Remarks

DR FULWYLER

The only comments to which I wish to respond are those of Dr Husain. It is quite true that the volume-measuring cell sorter has been used mostly with blood cells however at the University of Colorado the device has been extensively applied to dispersed mouse spleen cells and bone marrow cells The problems to be encountered with vaginal and cervical samples are much greater as Dr Husain points out, because of the presence of mucus, cell clumps which must be dispersed, and bacterial infection We are well aware of these problems To answer his question, we do not know at this point in time if cervical samples can be made suitable for processing by our method however efforts are underway the Los Alamos Scientific Laboratory and at the National Cancer Institute to resolve this point

Dr Kamentsky

In response to your request for closing remarks on Sorting Or Marking The Prescreened Cells' there is no basis for making closing remarks and I decline from doing so

M Meyer

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Chapter VIII Patterns of Cell Populations

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Opening Remarks

Dr. Thorell †

It is common to think that variations in a specific tissue cell population in terms of cell sizes, staining properties, etc. normally show rather narrow limits, but that pathologic disturbances (e.g. changes into malignancy) may cause considerable distortions, which can be expressed by anomalous distributional patterns, for instance histograms. This is generally expressed as "monomorphism contra "polymorphism," terms which are often used by pathologists, even in a graded fashion. However normal organ or tissue cell populations also exhibit variations in relation to state of development and/or function. For instance, the undifferentiated mesenchyme in the embryo represents a homogeneous cell population with regard to morphology as well as cytochemistry and functional properties When such mesenchyme determined but still undifferentiated, is exposed to induction as in the *in vitro* kidney mesenchyme system of Grobstein,[1] a sequence of events starts in the "committed" elements, leading to the formation of groups of cellular condensates which further develop into kidney tubules. Thus, with time an increasing degree of complexity is attained, which can be expressed in morphologic and cytochemical parameters. Plots of histograms, scatter diagrams, contour-maps etc can visualize degree and type of tissue intercellular variations and furnish information about state of development or function.

The opening remarks will briefly discuss some aspects of the informational content of distributional patterns obtained by plotting data from "mass-analysis of more or less randomly selected cells in tissues and body fluids.

The most simple example is furnished by the red blood cell population The single erythrocyte diameter has long been used as a parameter in plotting histograms or drawing the so-called Price-Jones curve The normal erythrocyte population exhibits a rather symmetrical and Gaussian distributional pattern Pathologic states such as pernicious anemia show characteristic deviations, useful for diagnosis

With the great numbers of cytochemical and cytophysical techniques available there is a wide choice of parameter descriptors or combinations thereof to use for the identification of single cell members in a population Relatively rapid more or less mechanized scanning cytophotometers have been developed so that an adequate number of cells can be analyzed within a reasonable time for various optical parameters the cells might be unstained or stained, living or fixed "Semiautomated" cytophotometric Feulgen DNA estimations are an example of how to collect data for visualizing the ploidy pattern of cell populations in the form of histograms.[1]

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Recently very rapid cell spectrophotometers has been constructed for measuring multiple optical properties of individual cells in liquid suspensions The rate of measurement exceeds 500 cells per sec. As all particular matter including single cells, cell clusters, non-cellular debris etc are measured, the question of how to prepare the cell population sample becomes critical. Depending on the method by which cell suspensions are prepared, especially from solid tissue, more or less selection will occur Such selection can be avoided in the conventional cytophotometric analyses of ordinary microscopic specimens, smears or sections, at least theoretically

The inevitable artefacts or errors inherent in various methods of sample collection, cytochemical preparation and micro-optical measurements are not necessarily obstacles preventing the usefulness of the recorded patterns of cell populations For the purpose of identifying functional differences in cell populations these effects might enhance separability of various states and on the basis of empirical results make discrimination and its functional interpretation more easy

So much for the technical aspects These have been amply treated in the preceeding parts of this Symposium

The information in plot, representing the pattern of cell population is evidently depending on choice of parameters and number thereof Again, the red cell system will be used as examples Figure 1 shows the distribution of erythrocyte diameters from normal individual and case of severe iron deficiency anemia There is no significant difference between the tw However if the amount of hemoglobin per single cell (cytophotometrically measured) is used as single parameter there is an easily distinguishable abnormality in the erythrocyte population from the iron deficiency case (Fig 2)

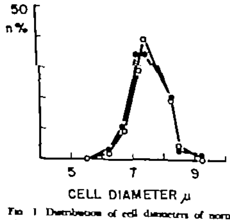

Fig 1 Distribution of cell diameters of normal erythrocytes (—○—) and erythrocytes from case or iron deficiency anemia (—●) N separability 1 ÷ 2

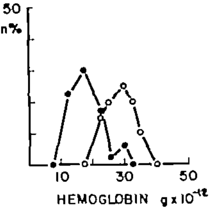

Fig 2. Distributions of hemoglobin contents per erythrocyte from normal human (—○—) and the case of iron deficiency anemia (—●—) Ref 2

This simply demonstrates that to distinguish between patterns of cell populations, even very homogenous ones, the choice of parameter is decisive This seems self-evident in the present example but it is not always considered Plenty of Feulgen histograms from various tumors have been published showing patterns of similarities or dissimilarities without any meaningful interpretations or even systematic relationships

The last mentioned shortcoming seems to rest on the circumstance that the increasing complexity of cell population needs corresponding higher "resolution in the plotted pattern to meet the requirement of specificity and eventual interpretability This can be illustrated as follow

The early developmental compartments erythropoiesis, corresponding to the morphologic stages of basophilic erythroblast and early polychromatic erythroblast are characterized by lively mitotic activity Within each of these compartments the cells are different with respect to stage in the mitotic cycle The Feulgen histograms for each compartment show values ranging from diploid to premitotic tetraploid, characteristic pattern for an intensely growing cell population (Fig 3) On the other hand, the late polychromatic erythroblast compartment has no growth function at all, and the Feulgen histogram depicted in Figure 4 displays the pattern of cell population homogenous with regard to diploid DNA-content per cell

The Feulgen histogram of the total erythroblastic cell population (i next higher degree of complexity) will obviously show an ordinary biased" histogram, not very specific and in which the dif ents are not readily resolvable

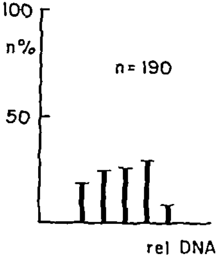

FIG. 3. Distributional pattern of Feulgen DNA per cell (*basophilic and early polychromatic erythroblasts*). Data from ref. 12.

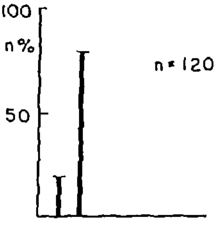

FIG. 4. *Distributional pattern of Feulgen DNA per cell.* Late polychromatic erythroblasts. (The pyknotic nuclei *give some spurious values apparently* lower than diploid.) Data from ref. 1[illegible]

Distinction between patterns, *representing different* functional states is not possible (Fig. 5). At least one more dimension (parameter) must be added to provide sufficient information. In the present example, the hemoglobin concentration of the single cell can be used as a second parameter plotted together with the Feulgen data, for instance in the form of a scatter diagram. The resulting pattern of the erythroblastic cell population will then contain new information based on the fact that the cells in the latest compartment *normally* have the highest concentration of hemoglobin. In this way, the scatter diagrams of Figure 6 *distinguish between three different states of* erythroblastic populations and might even allow some cell physiologic interpretations to be made.

For the diagnostic cytologist it is sometimes difficult or even impossible to pinpoint distinctive abnormal features based on the morphology of the single cell. In some instances mass-analyses and plots of the appropriate pattern of cell population might reveal characteristic differences. These possibilities are not yet much explored, but here is an example.

Lymphoid cells exhibit in many cases, even in malignancy, inconspicuous changes in cytology or *no definable alterations at all. The term* "atypical lymphocyte" is rather vague and not restricted to neoplasia, at least in the hematologic literature. Now, if a number of randomly selected cells in a normal lymphocyte population from lymphnode and/or peripheral blood is analyzed for certain properties, i.e., the cytoplasmic ultraviolet absorption and nuclear diameter, a linear relationship can be found between these two parameters.[illegible] The ultraviolet absorption properties in this case is represented by the quotient in absorption between *260 mμ* and *280 mμ* of cytoplasmic areas, which quotient under certain methodologic conditions indicates the relative RNA concentration.[11] The large lymphocytes have the highest quotient and

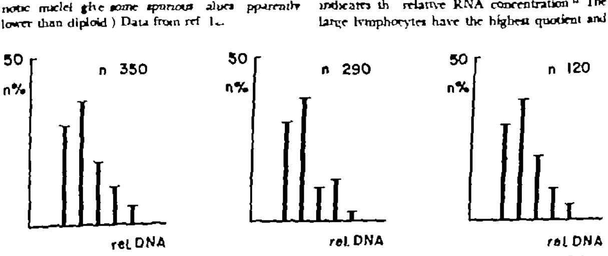

FIG. 5. Distributional pattern of Feulgen DNA per cell in the total erythroblastic population from (left to right) normal human bone marrow, erythroblastic *liver nodule in Rauscher virus infected mice, regenerating* mouse bone marrow after intravascular hemolysis (phenylhydrazin). No distinctions between patterns. Data from ref. 12 and 10.

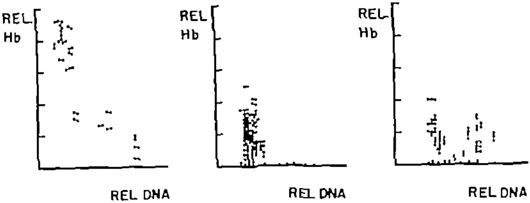

FIG. 6. Distributional patterns of hemoglobin versus Feulgen DNA per cell for same erythroblastic populations as in Fig. 5. In normal human bone marrow the highest hemoglobin concentrations are found in cells with postmitotic, diploid DNA values (late polychromatic erythroblasts). There is also rather uniform distribution between cells of different degrees of hemoglobinization with regard to phase in division cycle (inter mitotic phase). The erythroblastic Rauscher liver nodule cells exhibit hemoglobinization predominantly in elements with postmitotic DNA values. Few growing cells are engaged in hemoglobin synthesis. Erythroblasts from regenerating bone marrow show hemoglobinized cells in all stages of the mitotic cycle. N cells has tained the final degree of hemoglobinization. Data from ref. 12 and 10.

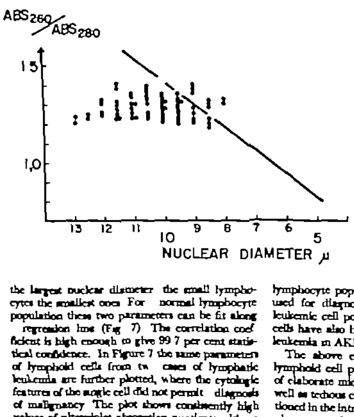

FIG. 7. Regression line (cytoplasmic ultraviolet absorption versus nuclear diameter) for normal lymphocyte population and same parameters plotted for cells from lymphatic leukemia. See text. Ref. 3 and 4.

the largest nuclear diameter the small lymphocytes the smallest ones. For normal lymphocyte population these two parameters can be fit along regression line (Fig. 7). The correlation coefficient is high enough to give 99.7 per cent statistical confidence. In Figure 7 the same parameters of lymphoid cells from two cases of lymphatic leukemia are further plotted, where the cytologic features of the single cell did not permit diagnosis of malignancy. The plot shows consistently high values of ultraviolet absorption quotients with no correlation to the nuclear diameters. Thus, the pattern of leukemic cell population reveals distinguished features compared with the normal lymphocyte population. Of course, this might be used for diagnostic purpose. Similar results on leukemic cell populations compared with normal cells have also been reported for the spontaneous leukemia in AKR-mice.

The above exemplified plots of erythroid or lymphoid cell population patterns are the results of elaborate microspectroscopic data collection as well as tedious computation and plotting. As mentioned in the introduction, very rapid microspectrophotometers have been constructed to cut down time for data collection considerably. In addition, the rapid microspectrophotometer was coupled with an interface to an IBM 1130 computer. This provided means to store the spectrophotometric data on magnetic disks, possibilities for on-line computation to develop various functions of the spectrophotometric properties and the capability to display these functions directly as histograms, contour-maps etc. I will not go into details of this rather preliminary work here but refer to the original paper.[8] The plots and also contour-maps representing cell populations with different functional properties showed in principle the same features as have been discussed with the examples above. However these trials involved for each sample measurements of about 30 000 cells within a few minutes, machine-computation and plotting of multi-dimensional "patterns of cell populations". Obviously much experimental work of empirical *type as well as the study of various* computational techniques and display methods is needed before analyses and visualization of "patterns" can be useful in automated cytology. But I look forward to seeing the discussions of these opening remarks.

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Discussion

Dr. Anderson

The concept that these are patterns of cell populations implies that distinct and distinguishable cell types exist. That this is true morphologically is one of the major contributions of cytology. *However rather little* consideration has been given to the implication of this fact at the molecular level. In this paper I would like to discuss how cell populations may ultimately be defined.

Cells vary in composition both quantitatively and qualitatively. From simple control theory it is *evident that the level of each macromolecule cannot* be controlled separately; rather sets of proteins must exist which are turned on or off as a unit (separate regulatory mechanisms exist which may control the level of a protein or its activity). In the limiting instance a set may contain only one protein, but usually contains several. Several different types of sets are postulated to exist and include first and foremost the base set. This set is composed of all macromolecules found in all nucleated cells of a given species. The base set obviously contains DNA, a set of metabolic enzymes, the minimum complement of proteins required for cell survival, and the RNA associated with their production. The base or *B set* can be studied in a number of ways, the most convenient, for proteins, using fluorescent-labeled antibodies.

The remaining macromolecules may from this view be divided into differentiation or *D sets* which by definition are not found in all cells. D sets are of several types depending on the restric*tions governing their appearance*. D sets may be divided into groups of sets which may be contem*poral in the cell, i.e. one set does not contain a* repressor for another. In contrast, non-cotemporal sets cannot exist at the same time. Sets may be sequentially dependent (if A, then B) and may be arranged in linearly dependent linkages which include both positive and negative dependencies (if A, then B, if B then C if A is absent). Sequences of this type allow for gradual gradation and timing. For example, if B in the last example contains a repressor of A, and set A contains a repressor of C, then when B appears, the appearance of C will be delayed until set A has disappeared.

Description of differentiation and of cell populations in terms of known molecular sets will occupy our attention for many years. A number of sets may be identified at the present time. These include, for example, α and β crystalline of lens, hemoglobin in erythrocytes, and the auto-antigenic basic protein of neuronal tissue. While much information may be gleaned from literature surveys, exhaustive studies of the distribution of well characterized single molecular species (single isozymes, for example) are difficult to accomplish. To demonstrate that two proteins belong to the same D set requires not only proof that they can coexist, it must be shown that they always coexist, I are turned off and on together

Differentiation and much of cytopathology will ultimately be described in terms of Markov chains which include large number of sets as elements. If this is true, it is important that not only the occurrence of specific enzymes or antigens in tissues be well documented, but also their absence.

Drs. Bartels, Bahr and Wied

Dr Thorell touches upon two questions of fundamental nature First, the problem of selection of suitable dimensions, or "features" in a pattern space is taken up One may examine substantial number of cell properties sequentially and assess their discriminatory potential one by one One may for example, compute for each an information measure based on the probability density functions found for the members of both pattern classes (or cell types) to be discriminated One can then decide which dimensions yield best discrimination, and select restricted number for classification purposes. This method is workable. Its main disadvantage, that the covariance between different cell properties is not fully exploited, could at least be partially remedied by a subsequent processing step

There exists rich literature concerning strategies for feature selection

It is definitely not advisable to use more dimensions than absolutely required The higher the dimensionality of the pattern space, the less degrees of freedom are available for multivariate statistical significance test, on the basis of which difference between populations would have to be established Also, the risk of observing spurious information resulting from random sample divergence increases sharply

There is no general solution to the problem It is not possible to predict which cell properties might best discriminate two given sets of cells, so that an exhaustive search is presently the only answer

The second problem touched upon by Dr Thorell's opening remarks concerns the homogeneity of cell population The examples given by Dr Thorell all involve cell populations which in themselves are relatively homogeneous Let us then assume cell population which is rather homogeneous Let it furthermore be assumed that the sample size is considerably larger than the dimensionality of the pattern space, so that good estimate of the samples' covariance matrix is available This covariance matrix then represents the population pattern

A different cell population has different covariance matrix—different means, eigenvectors, and eigenvalues, and these differences define the position of an optimized decision boundary between the two cell populations.

These straightforward conditions, however are not given in the case of material from the female genital tract.

A cell sample here consists not of one but several cell types. Let it be assumed that the relative frequencies of occurrence are constant. Each of these cell types is characterized by probability density distribution, and most of these will overlap within the pattern space.

The cell population to be discriminated from this irregularly distributed cell sample consists essentially of precisely the same elements. The cell types are the same, their proportions shall even be assumed to be similar—but the abnormal sample contains small proportion of outliers It is these outliers, and their detection within the ill-defined heterogeneous multivariate distribution which is of concern here.

No doubt one can find such best linear combination of the original dimensions, along whose direction the probability density assumes maximum difference somewhere for normal sample, and sample which contains abnormal cells

One may envision these multivariate distributions somewhat like point cloud, comparable in shape to the strange balloons which can be bought at country fairs, which sport rabbit ears and paws. The cell sample with the abnormal cells in it would have an additional little bulge somewhere. The linear combination sought is a direction which goes straight through this bulge where it has its greatest difference to the normal rabbit balloon The question is then reduced to one-dimensional problem. One now tries to distinguish between two distributions which are essentially identical, but one expects a small probability density difference in certain region along the composite parameter' axis.

Whether now population pattern is significantly different from normal control comes down to significance test for the difference between two distributions, one of which has the expected density values for normal population, the other the expected values for population with abnormal cells in it

In fast devices, such as flow systems, no difficulty should exist for working with more than adequate sample sizes

Dr. Tolles.

It is very rewarding to find distinguished biophysical scientist proposing discussion of quantitative properties of cells and the importance of the dimensionality of such quantitative descriptions. The field of automatic cell recognition has much to gain from such considerations.

Thorell illustrates well the ineffectiveness of a non-contributing parameter (cell diameter in this instance) to distinguish between the erythrocytes from a normal subject and those from a subject having severe iron-deficiency anemia. A more *discriminating parameter is shown to be the* cellular hemoglobin content though considerable overlap exists between the two populations. This example is analogous to many one-dimensional studies typified by Cowdry[1] and Reagan[6] and which were very influential in leading contemporary quantitative thinking. These methods showed effectiveness in distinguishing between *two different groups of cells although complete* separation of the types of cells studied was not achieved.

Thorell then shows the increased discriminatory power of a two-dimensional decision process in comparison to the power of either of the parameters alone citing nuclear size and ultraviolet absorption ratio, and RNA and DNA as parameter pairs. Though more discriminative, overlapping of the groups of cells was not avoided.

Tolles[8] employed three-dimensional scatter groups comprised of cell diameter, nucleus diameter and nucleus extinction coefficient which clearly *delineated the island of abnormal neoplastic* cells among the broad distribution of normal cells. Though complete separation was not achieved, a much higher degree of isolation was attained than with lesser dimensionality. Prewitt and Mendelsohn,[5] also employing three independent descriptors, were able to divide white blood cells into four groups with but 3 per cent intergroup overlap. Thus, the inculcation of each additional meaningful parameter strengthens the discriminating power of quantitative descriptions of classes of cells.

More recently McMaster[4] (employing photomicrographs of 29 cells) used five parameters chosen from the *cytologist's diagnostic language* hyperchromasia, granularity, irregularity of nuclear outline, nuclear size and eccentricity. Not surprisingly he was able to achieve complete separation between normal basal cells and malignant cells since the number of parameters was not greatly different from the number of cells. Application of his method to a large number of other than easily defined cells is anticipated with interest. Wied, Bartels, *Bahr[11] et al., also employing* small numbers of cells, used variously several morphologic and densitometric parameters: maximum extinction, EPERC, cell size, nucleus size and a *hypergeometric* measure with encouraging results.

This background shows convergence to the analysis of cell patterns by means of cluster theory or some form of multi-variable probability formulation commonly used in problems with large numbers of variables (parameters) such as found in electrocardiography *as described by Tolles.*[10]

Current attempts to mechanize or automate a pre-screening device are evenly divided between low resolution flow instruments (Kamentsky[3]) for the measurement of bulk properties such as cell volume, *D*NA, RNA and high resolution scanning or image dissecting methods (Gusberg,[2] Rosenberg[7]). In either case, only a small number of parameters is dealt with. None have yet shown clinical value, the problems being two-fold: inability to accept a high percentage of specimens, and a high false positive rate. The difficulty does not seem to be the detection of abnormal cells, *but rather inadequate collection and preparative* techniques and the overwhelming presence of cellular aggregates falsely qualifying as suspicious cells according to the parameters used.

Thus, successful automation of cytologic screening may reside in discovering more meaningful dimensions of decision but it will certainly be potentiated by some means of segmentation of the cellular specimen to reduce greatly the opportunity for false positive indications without encountering a severe loss of original specimen.

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Closing Remarks

Dr. Thorell

I am grateful to the discussants, who in many essential points have thrown light upon the topic "Patterns of Cell Populations" Dr Andersson brings this concept down to the molecular level which, of course, constitutes the fundament for the characteristics of and changes in pattern during growth and differentiation. His "set analysis" can with advantage be applied on already existing cytochemical data, for instance, the changes in pattern of cell population during differentiation of the embryonic nephrogenic mesenchyme in terms of cellular nucleic acids and the successive appearance of various enzymes in condensate and tubular cells (Sundelin, *et al.* Exp. Cell Res. 54 347–52, 1969 Saxén Int. Rev Exp. Pathology Vol. 8, 1970)

Drs. Bartels, Bahr and Wied view the problem from a more practical point, i. e., cancer detection. Certainly there is no general solution to the problem which cell properties might best discriminate populations of normal and abnormal cells. Much downright empirical data collection on well defined clinical material is necessary before any practical application can be foreseen. Recent although preliminary data give some hope, however (Zajicek *et al.* Acta Cytol (in press), Kamentsky and Thorell Acta Cytol 14 307 12, 1970)

Dr Tolles is concerned about the technical-preparative difficulties for obtaining cellular specimen suitable for existing and future automatic screening devices. From own experience I feel more optimistic Moreover for certain types of specimen, computer-handling of two or more parameters-descriptors might cancel false effects arising from cellular aggregates (see ref above)

Finally with reference to the discussion on "Other Parameters Descriptors to be Considered" If we are going to live with machines and automated cytology we must by careful experimentation learn more about how the machines look upon cellular specimens and what they can and cannot distinguish as normal and abnormal.

dimensionality of such quantitative descriptions. The field of automatic cell recognition has much to gain from such considerations.

Thorell illustrates well the *ineffectiveness of a* non-contributing parameter (cell diameter in this instance) to distinguish between the erythrocytes from a normal subject and those from a subject having severe iron-deficiency anemia. A more discriminating parameter is shown to be the cellular hemoglobin content though considerable overlap *exists between the two populations. This* example is analogous to many one-dimensional studies typified by Cowdry[1] and Reagan[6] and which were very influential in leading contemporary quantitative thinking. These methods showed effectiveness in distinguishing between two different groups of cells although complete separation of the types of cells studied was not achieved.

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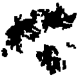

FIG. 1 Quinacrine hydrochloride stained Y chromosome within an intermediate cell nucleus from the buccal smear of a normal male. More bright spots in the nucleus near eight o'clock. Leitz Ortholux fluorescent microscope dark field illumination rephotographed from Ektachrome high speed film (×3,600)

Mrenova and Albrecht[14] there is more resistance to fading at higher pH values. Fothergill[12] says that with fluorescein conjugates maximum fluorescence is reached at pH 8.0, but Nairn *et al.*[16] suggest that a pH of 8.6 may be optimal. Quinacrine appears to behave similarly in respect to pH. The advantage of an alkaline mounting medium can be explained by the observations of Zharos and Zolotukhin[18] who reported that atabrine is an "acid-base luminescence indicator" whose fluorescence under ultraviolet light changes from "faint green" to "bright green" at approximately pH 7.0

Caspersson and his co-workers[8] reported that quinacrine mustard binds to DNA about 20 times more strongly than quinacrine hydrochloride (*Vicia faba* chromosomes at pH 4.1) and concluded that the mustard compound is superior for chromosome studies because of its alkylating action. Quinacrine mustard stained chromosomes were analyzed with a photoelectric technique and recently fluorescence patterns for each of the individual human chromosomes have been described. We have also found that quinacrine mustard gives good results with our modified staining procedure.

SUMMARY

An improved technique for staining the human Y-chromosome by quinacrine hydrochloride is described. Increased fluorescence and stability is produced by utilizing an alkaline mounting medium. This provides a reliable stain for the study of details of Y-chromosome structure and renders the Y-chromosome easily available for rapid morphologic identification and enumeration.

ACKNOWLEDGMENT

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The Quinacrine Fluorescence Method of Y-Chromosome Identification

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Relatively specific fluorescent staining of the human Y-chromosome has recently been accomplished[1,7,10,11,17,18] with methods based on the observations of Caspersson *et al.*[3] that metaphase chromosomes of *Vicia faba* and of *Trillium erectum* contain DNA areas which bind certain fluorochromes. The distal end of long arm of the human Y-chromosome can be stained by quinacrine hydrochloride (atabrine) or by quinacrine mustard. Zech[1] Caspersson *et al.*[7] and George[13] reported fluorescence of human Y-chromosomes in metaphase plates. Caspersson *et al.*[8] and Pearson *et al.*[17] stained Y-chromosomes in interphase nuclei and Barlow and Vosa stained Y-chromosomes in human spermatozoa.

This laboratory has confirmed the presence of quinacrine-stainable human Y-chromosomes in metaphase plates, in interphase nuclei, and in spermatozoa. We have also studied abnormal Y-chromosomes, noting as an exception that the short Y-chromosome[4] does not stain.

Because prior methods[11,7,19] gave transient and relatively unsatisfactory stains with quinacrine hydrochloride, we modified the staining technique. This communication is to describe our modified staining method which clearly and consistently demonstrates the quinacrine-stainable Y-chromosome and is relatively long lasting.

METHOD

Fix smears without drying in 95% ethyl alcohol	1 hour (longer)
Methyl alcohol	
Graded ethyl alcohols to water	3 minutes 3 minutes each
0.5% aqueous quinacrine chloride	5 minutes (or longer)
Distilled water 2 changes	3 minutes each
0.01 M citric acid phosphate buffer (pH 5.5)	3 minutes
0.01 M phosphate buffer (pH 7.4) 2 changes	3 minutes

Mount with 0.01 M phosphate buffer (pH 7.4) and seal with clear nail polish.

Examine with fluorescent microscope and dark field illumination.

OBSERVATIONS

Various methods of fixation were satisfactory. 95% ethyl alcohol followed by secondary fixation in methyl alcohol allowed excellent staining equal to that obtained with Papanicolaou's ethyl alcohol-ether mixture. Y-chromosomes also stained well in restained air-dried preparations originally fixed with acetic acid-alcohol and stained with Giemsa or hematoxylin. Staining solutions prepared by dissolving and filtering pharmaceutical atabrine hydrochloride tablets (Winthrop) were indistinguishable from those made with pure quinacrine hydrochloride (Winthrop). Differentiation in 5.5 pH buffer produced good stains while prolonged exposure at 5.5 pH caused loss of the Y-chromosome stain; however, the optimum times and pH levels for differentiation remain to be determined. Similarly, a pH of 7.4 is not necessarily at the optimal level for alkalinization and mounting. Stained slides remained satisfactory for several days. Dark field illumination was best for microscopic study, but incident illumination (Leitz "Orthoplan") was also satisfactory.

Well preserved normal male interphase nuclei contain a single well defined fluorescent spot (Fig. 1) randomly located within the nucleus. In metaphase plates a brightly fluorescent area is located at the distal end of the long arm of the Y-chromosome and fainter fluorescent areas are also present on other chromosomes.

DISCUSSION

The crucial change in our modified stain is that we have increased and stabilized fluorescence by stipulating an alkaline final pH. Nairn[1] has discussed the advantages of an alkaline mounting medium in the case of fluorescein. With fluorescein conjugates, according to Petal and Janowitz, there is enhanced fluorescence and according to

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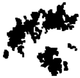

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METHOD

Fixation without drying in 95% ethyl alcohol	*1 hour (or longer)*
Methyl alcohol	3 minutes
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Comparative Sequential Cytologic Changes Following in Vitro Infection with Herpesvirus Types I and II

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Increasing reports of herpes infection of the human genital tract attest to its growing recognition and importance. There is suggestive evidence for an association between genital herpes infection and carcinoma,[1-5] and there are chronic virus infections followed by immunopathy[6] and effects of many viruses upon chromosome structure.[7-9] These data require that more attention should be directed toward the long-term effects of virus infection. The sequential cytologic manifestations of cervical infections are therefore of primary importance. A number of excellent clinical studies has been reported, but they are necessarily nonsequential and diagnostically uncertain until or unless virological studies are made. In order to understand more clearly the temporal pattern of events in herpes-virus infection, the practicality of an *in vitro* model system was investigated.

Three different human cell cultures were simultaneously infected with herpes simplex virus (HSV Type I) and herpes genitalis virus (HGV Type II). Both virus stocks were kindly supplied by Dr. Bertuna B. Wentworth, Department of Epidemiology and International Health, University of Washington, Seattle.

MATERIALS AND METHODS

Virus Production

Virus propagation was accomplished in HeLa cell cultures at an input of 10^4 plaque forming units (PFU)/ml. Usually 24 to 48 hours were necessary for the beginning of cytopathic changes, which were complete after 96 hours. The culture medium was minimum Eagle's medium (MEM) supplemented with 2% fetal bovine serum (FBS). Seventy-five cm² plastic flasks were used and cultures maintained at 37° C.

It was found that a new cell line ME180[10] also supported the passage of both viruses and was used subsequently as an additional host-cell for virus production.

Cell Cultures

1. #200. This was derived from the skin biopsy of a 9½ year old female and established in our laboratory. It is a normal cell, by karyotype, with a mean generation time (MGT) of about 26 to 28 hours. It was free of mycoplasma by culture.

2. HeLa. This line has been kept in our laboratory for the past six years, originally obtained from Dr. J. Sisken. The modal chromosome number is about 65 with a range of 52 to 142. This line was free of mycoplasma and had a MGT of about 18 hours.

3. ME180. This was derived from a metastasis of cervical carcinoma. This cell line was obtained from its originator, Dr. J. Sykes.[10] It had carried, apparently symbiotically, *M. hyorhinis* which was resistant to kanamycin treatment. The modal chromosome number was 65 with a range of 48 to 130. A tendency to form multinucleated giant cells is a constant feature. It retains a high degree of differentiation, being X-chromatin positive and producing tonofibrils and keratin, the latter when on low serum concentration. Its MGT was comparable with HeLa, about 18 to 20 hours. All cultures were grown on MEM supplemented with 10% FBS at 37° C.

On the starting day, duplicate cultures were treated as follows: each culture was inoculated with 10^6/ml cells. Except for the controls, the remaining (experimental) sets were infected with either HSV or HGV and duplicate cultures harvested for cytology every 24 hours.

Cells for harvest were washed twice with Dulbecco's phosphate-buffered saline (PBS), then gently scraped from the flasks with a rubber policeman, collected in PBS and concentrated by centrifugation. Cells were deposited on albumen-rubbed slides by a bacteriologic loop or by centrifugation in a cytocentrifuge. They were fixed and stained by standard Papanicolaou procedures.

RESULTS

Normal controls were harvested each day in order to monitor possible cellular changes occurring with time. This precaution proved unnecessary as there were no significant cytologic changes in controls during the period of the experiment.

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plasm is delicately thin and transparent. Nuclei were much enlarged, with central clearing (almost vesiculation) and an occasional inclusion body was visible. Multinucleation was frequent, apparently the result of cell fusion, although endoreduplication or endomitosis could not be eliminated as contributory.

(2) ME180. About 35 to 50 per cent of the cells showed slight, but definite, nuclear enlargement with central clearing. A beaded chromatin pattern occurred at the margins and there was some "molding" in multinucleated cells. It was difficult to be certain whether multinucleation had increased above control levels.

3 HeLa. Thirty to forty per cent of cells, mostly the small type in this polymorphous culture, showed changes comparable with those described for ME180. The central chromatin appeared homogeneous as well as more lightly stained ("frosted glass"). Cytoplasmic staining was somewhat deeper with increased opacity, possibly associated with protein and RNA synthesis occurring with the early stages of virus production.

96 Hours

1 #200. Aside from a tendency toward cell rounding, the changes were an extension of those at 72 hours. More degenerative changes appeared with loss of sharp nuclear and cytoplasmic borders, the appearance of a granular cytoplasm, and loss of sharp staining qualities.

2 ME180. Almost all cells were affected by this time. Nuclear vesiculation was marked and occasional intranuclear inclusion bodies could be seen. Nuclear molding was also prominent. Cytoplasmic vacuolation appeared for the first time.

3 HeLa. Virtually all cells were affected. The findings were essentially an extension of those at 72 hours. However, cell degeneration was also present at this time.

HSV Type II (Figs. 2a and b)

The first 24 hours revealed no significant alterations, although there may have been slightly more multinucleation in the #200 culture than in the controls (3% vs. 5%).

48 Hours

1 #200 Skin. Less than 1 per cent of cells showed eosinophilic homogenization of nuclear chromatin. Otherwise they were comparable with controls.

2 ME180. Approximately 80 per cent of cells were affected. Nuclear vesiculation with peripheral chromatin beading was the most common alteration. Next in frequency was the loculation of the nucleus by what appeared to be strands of chromatin. A barely perceptible nuclear inclusion could be detected in about 10 per cent of cells.

3 HeLa. Eighty to ninety per cent of the "large" cells of this culture showed striking loss of cytoplasm and loculation, homogenization or granulation of nuclei. "Small" cells of the culture exhibited nuclear condensation and homogenization and slight cytoplasmic vacuolation.

72 Hours

1 #200 Skin. By this time most of the cells were affected. Enlargement of the cells was accompanied by eosinophilic nuclear granulation. A finely dispersed cytoplasmic vacuolation was also noted. Increased multinucleation, degeneration of some cells and agglomeration of what appear to be nucleoli completed the picture of cellular changes.

2 and 3 ME180 and HeLa. The cellular changes at 48 hours were extended.

96 Hours

1 #200 Skin. More extensive changes occur at this time and nuclear loculation appears. Some intranuclear inclusions are present, but are not common (<20%).

2 and 3 ME180 and HeLa. Both cultures show extensive degenerative changes in addition to cytologic features already described.

DISCUSSION

This experiment emphasizes a difference in susceptibility among cell cultures for Type I and Type II virus, a fact which is undoubtedly reflected in clinical practice. The order and rapidity with which the susceptible cell develops morphologic disturbances varies between cells and again depending upon whether Type I or II is the infecting agent. Thus, while skin is most susceptible to Type I, it is least susceptible to Type II. Cytoplasmic changes were more striking when the skin cells were infected, possibly because these *were normal at the start, while both HeLa and* ME180 were derived from malignancies and characterized from the outset by heightened RNA and protein synthesis. Type II virus caused more rapid involvement of HeLa and ME180 than HSV and cells demonstrated a delicate nuclear loculation, a feature not encountered in these cell lines infected with HSV.

Both the cytologic manifestations and the rate of their development are distinguishing features between Type I and II infections. It may be possible to finally determine morphologic characteristics which will permit definitive interpretation. However, these investigations re-emphasize the fact that nuclear changes occur before inclusion bodies appear. Even at the end stage of infection only

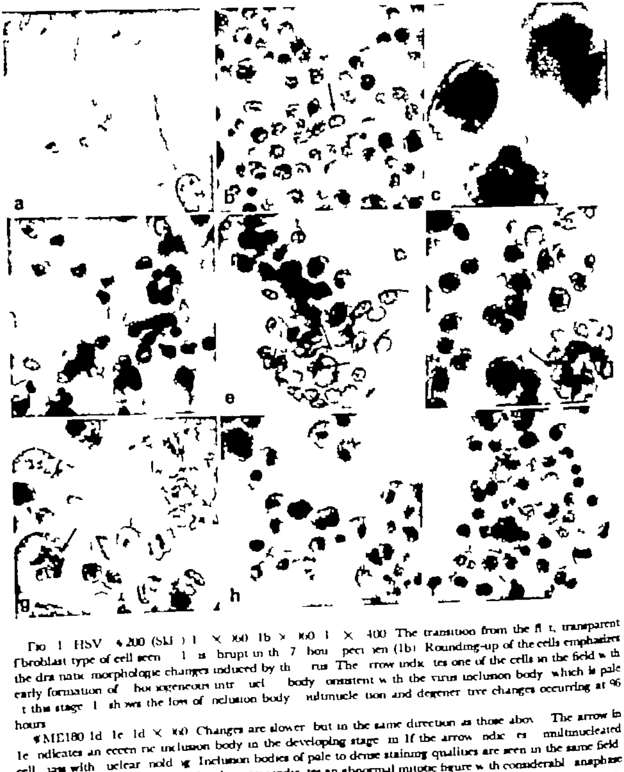

FIG. 1. HSV #200 (SK) 1a × 400 1b × 400 1c × 400 The transition from the flat, transparent fibroblast type of cell seen in 1a is abrupt in the 7 hour specimen (1b). Rounding-up of the cells emphasizes the dramatic morphologic changes induced by the virus. The arrow indicates one of the cells in the field with early formation of homogeneous intranuclear body consistent with the virus inclusion body which is pale at this stage. 1c shows the loss of inclusion body, multinucleation and degenerative changes occurring at 96 hours.

#ME180 1d 1e 1f × 400 Changes are slower but in the same direction as those above. The arrow in 1e indicates an eccentric inclusion body in the developing stage. In 1f the arrow indicates multinucleated cell mass with nuclear molding. Inclusion bodies of pale to dense staining qualities are seen in the same field.

#HeLa 1g 1h, 1i × 400 In 1g the arrow indicates an abnormal mitotic figure with considerable anaphase lag. 1h shows that more cells in the culture have been converted to the small rounded type than are present in the control similar to the change occurring in skin (1a → 1b). Pale intranuclear inclusion bodies (difficult to photograph) are present at the 96th hour.

HSV-type I (Figs. 1a and b)

During the first 48 hours, there were no detectable differences between the control and experimental sets.

7 Hours

(1) Skin. Almost all cells (80%) were affected. Most notable was the deep staining and opacification of the cytoplasm. Normally this cell's cyto-

curred more frequently with Type I than with Type II infections.

It seems apparent from past descriptions (and our own experience) that either virus may be implicated in genital infections. At this moment, it is not evident whether the infection is at last being recognized or whether there is actually an increase in the frequency of occurrence. In addition to the possible role in cervical cancer it is worth speculating whether some of the recurrent urogenital infections of obscure origin may not actually represent herpes infection.

Conclusions

1 Cytologic events following herpesvirus infection can be adequately studied using an *in vitro* system.

2. Herpesvirus Types I and II infections can be distinguished when cell cultures are infected Type II causes nuclear loculation not seen in Type I infections.

3 Intranuclear inclusion bodies are late and statistically minor manifestations of herpesvirus infections

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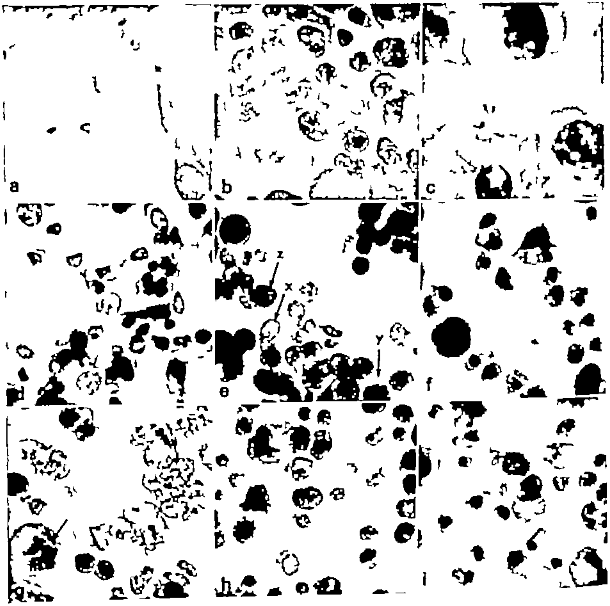

FIG 2. HGV #200 (Skin) 2a × 960 2b × 960, 2c × 400. Again, as in the HSV experiment, no significant changes occur until 72 hours, but the cells tend to remain more flattened and larger than with HSV. Multinucleation and opacity of the cytoplasm are well developed. In 2c the reticulated character of the nuclear chromatin is developed. The 96 hour specimen is better preserved than with HSV showing that the course of the Type II is slower.

#ME180 2d, 2e 2f × 960 2 represents the 48 hour specimen, showing that in cervically derived tissue the Type II infection proceeds more rapidly than the Type I (present also with HeLa, but not shown). Arrow Y the pronounced reticu- X shows the central clearing of the nucleus with peripheral beading of chromatin, lated chromatin pattern resulting in nuclear loculation, and Z the early formation of nuclear inclusion bodies. By 96 hours (2f) the cells have become small and round or multinucleated and the nuclei densely staining.

#HeLa 2g 2h 2i × 960 2h (72 hours) again demonstrates the effect of virus on morphology most cells being smaller than in the control culture. Multinucleation and reticulation of nuclear chromatin are evident here and in the 96 hour specimen. Inclusion bodies have not been emphasized. They are cytologically important, of course but represent relatively late and statistically infrequent changes.

comparatively small percentage of cells exhibit clearly defined inclusions. Central nuclear clearing beading of nuclear borders and loculation are the key changes of the nucleus, in Type II infection. Cytoplasmic changes are deepening of stain, opacification and finally vacuolation. Loculation of the nuclei helps distinguish between Type I and II infections. Intranuclear inclusion bodies oc by sputum cytology It should be noted, however that at our Center the TV-brushing technique has been mainly applied to subjects that had negative sputum cytology or did not produce any sputum. The positive rate of sputum cytology in our laboratory during the past five years was 70.7 per cent (435 positive cases out of 615 patients with confirmed primary lung cancer)

TABLE I. *Results of the TV-brushing technique for peripheral lung lesions*

Final diagnosis	Total number of cases	Total number of positive cytology cases by TV brushing technique
Primary lung cancer	208	172 (82 7%)
Metastatic lung cancer	18	12 (66 7%)

Cytology of fresh cancer cells obtained by the TV brushing technique

Adenocarcinoma (Table III) Cancer cells obtained by the TV-brushing technique are found as cell-groups (clumps) on the smear However acinous structure of cancer cells is rarely observed whereas irregular cellular arrangement in cell-groups is frequently seen. Cellular rims (borders) of fresh adenocarcinoma cells are well defined in 21 7 per cent of 1,589 cells, poorly defined in 62 5 per cent and not stained in 15 7 per cent Nuclei of fresh adenocarcinoma cells are mostly round or oval in shape (77 6% of 1,589 cells) and nuclear borders are generally thin (97 3%) Chromatin patterns of adenocarcinoma cells show fine reticu- r and or fine granular in 92 2 per cent of 1,589 lls, coarse reticular in 1 4 per cent and coarse anular in 6.5 per cent Nucleoli of fresh adeno- rcinoma cells are usually large (89 9% of 970 lls) round in shape (90 8% of 970 cells) and ostly situated near the center

As shown in Figures 2, 5, 6 and 7 even in very esh adenocarcinoma cell-groups obtained by e TV-brushing several chromatin patterns of mor cells can be often seen In contrast, non- alignant cell groups obtained by TV-brushing

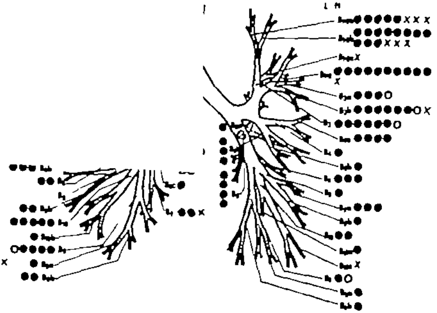

FIG 1 Result of the TV-brushing technique by bronchus. ● positive cytology with successful insertion of brush ○ positive cytology with insufficient insertion of brush X negative cytology

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Early Diagnosis of Small Peripheral Lung Cancer—Cytologic Diagnosis of Very Fresh Cancer Cells Obtained by the TV-Brushing Technique

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It has been generally accepted that an accurate diagnosis of small peripheral lung cancers (lesions less than 2 cm in diameter) has great value for early surgical treatment, but the positive rate of sputum cytology remains quite low for small cancers situated in the peripheral regions of the lung. Poor exfoliation from the small peripheral lesions and a degeneration of exfoliated cells in bronchial airways may be responsible for the low positive rate of sputum cytology for these small peripheral lung cancers.

To overcome the difficulties in establishing a cytologic diagnosis of small peripheral lung cancers, bronchial brushing or biopsy techniques have been developed.[1-4, 9, 11, 12, 13] We have developed a selective bronchial brushing technique under X-ray television fluoroscopy (TV brushing technique) in order to obtain fresh tumor cells directly from peripheral small tumors.[4-8] As described previously, cancer cells obtained by the TV brushing technique are very fresh and mostly in groups. However, morphologic characteristics of the malignant cells thus obtained are considerably different from those of exfoliated cancer cells in sputum cytology. For example, adenocarcinoma cells derived from a small peripheral lesion (less than 2 cm in diameter) do not necessarily show morphologic changes of malignancy as anisokaryosis, condensation of chromatin, thickness of nuclear border and indentation of cell border, etc. Therefore we mis-diagnosed two false positive cases and several false negative cases by the TV brushing technique during the past eight years. Presented in this report are the findings obtained by precise studies made to clarify the specific morphologic features of fresh cancer cells, especially adenocarcinoma cells, obtained by the TV-brushing technique.

MATERIALS AND METHODS

As reported previously,[4-8] in the TV-brushing procedure a straight brush and two kinds of flexible brushes were used: a flexible brush having an elbow-like joint near the tip and that (double-flexible brush) having two joints between the brush and wire. The material obtained by TV brushing was smeared on glass slides and then stained according to Papanicolaou's method. In some cases, phase contrast microscopy observation of the cells was conducted without staining and after fixing and staining by Papanicolaou's method the same cell-groups were observed by light microscopy.

RESULTS

TV-brushing technique for peripheral lung lesions

A total of 436 peripheral lung lesions, especially small lesions suggestive of lung cancers, were brushed by this technique. The final diagnoses of the cases subjected to TV-brushing are shown in Table I. Of the 208 confirmed cases of primary lung cancer, 172 cases were found to be cytologically positive by this technique for a positive rate of 82.7 per cent. On the other hand, two out of 136 cases of tuberculosis proved to be false positive by this technique. In these cases cells from the bronchial hyperplasia or tuberculous lesion were misdiagnosed as adenocarcinoma cells, as reported previously in this Journal.[7]

Results of the TV-brushing technique by bronchial region are shown in Fig. 1. As shown in this figure, the TV-brushing technique is effective for all bronchial ramifications.

Comparison of efficiency of the TV-brushing technique with that of sputum cytology in the diagnosis of lung cancer

The efficiency of the TV-brushing technique was compared with that of sputum cytology in diagnosing 208 confirmed cases of primary lung cancer (Table II). Of these 208 confirmed cases of primary lung cancer 82.7 per cent proved to be positive by this technique and only 10.1 per cent

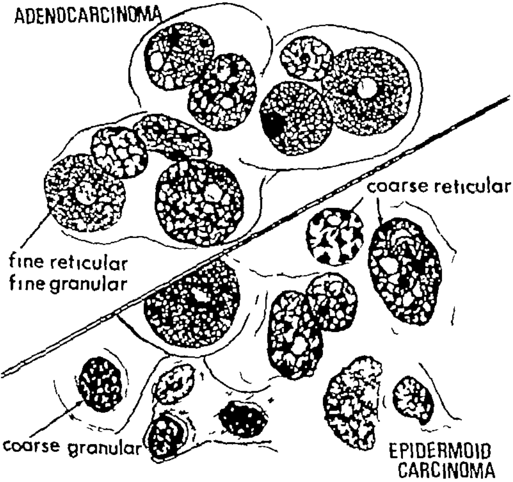


Fig. 2. Schematic drawings of fresh adenocarcinoma cells and epidermoid carcinoma cells obtained by the TV-brushing technique. Normally very fresh adenocarcinoma cells show fine reticular and/or fine granular chromatin patterns, while slightly degenerated cells show coarse reticular or coarse granular chromatin patterns. However, even in very fresh adenocarcinoma cell groups, several patterns of nuclear degeneration can be seen. This is very important finding being different from non-malignant cells. In fresh epidermoid carcinoma cell groups this finding is more remarkable: nuclear borders are rather thick and nucleoli are irregular in shape, and many cells having coarse reticular or coarse granular chromatin are mixed.

Epidermoid carcinoma. Nuclear borders of epidermoid carcinoma cells are rather thick and nucleoli are irregular in shape. The cellular rim is well defined and several patterns of nuclear degeneration in the same cell-group are frequently observed (Fig. 2). Therefore, diagnosis can be easily made by ordinary cytologic criteria.

DISCUSSION

It is 18 years ago that we commenced cytologic diagnosis of lung cancer. During the initial period, though it was possible to diagnose cases of advanced lung cancer beyond therapy, a defini diagnosis of small lesions which cannot be differ entiated from small tuberculoma could not b established. In 1962 with the introduction of th TV-brushing technique, it has become possibl to diagnose lung cancer of lesions about 2 cm size. However, as described in previous paper and in the Results, we observed fresh cance cells showing considerable different morphologi findings from those of exfoliated cancer cell

TABLE II *Comparison of efficacy of the TV-brushing technique with that of sputum cytology in the diagnosis of the primary lung cancer*

	Size of tumor*					Atelectasis	Total
	<2.0 cm	2.1-3.0 cm	3.1-4.0 cm	4.1-5.0 cm	>5.0 cm		
TV-brushing							
positive	9 (69.2%)	33 (66.0%)	47 (90.3%)	33 (91.6%)	47 (87.0%)	3 (100%)	172 (82.7%)
negative	4	17	5	3	7	0	36
Sputum cytology							
positive	0	2	4	4	7		19 (10.1%)
negative	9	40	44	30	46	1	170
not examined	4	8	4	2	1	0	19

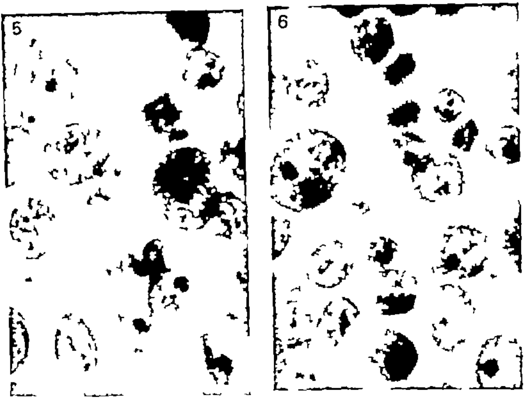
Maximum diameter of the tumor was measured on the routine PA chest X-ray film.
Three repeated examinations
Includes cases whose sputum was not examined or not available.

TABLE III *Morphologic findings of adenocarcinoma cells obtained by the TV-brushing technique from peripheral cancer less than 3 cm in diameter (1,589 cells in 19 cases)*

Cellular arrangement in cell group found on smear		acinous arrangement	1/19 cases
		irregular arrangement	13/19 cases
		acinous and irregular arrangement	5/19 cases
Cellular rim		well defined	344/1,589 cells (21.7%)
		poorly defined	995/1,589 cells (62.6%)
		not stained	250/1,589 cells (15.7%)
Shape of nucleus		round or oval	1,234/1,589 cells (77.6%)
		irregular	355/1,589 cells (22.4%)
Chromatin pattern		fine reticular and/or fine granular	1,465 cells (92.2%)
		coarse reticular	22 cells (1.4%)
		coarse granular	102 cells (6.4%)
Nuclear border		thin	1,546 cells (97.3%)
		thick	43 cells (2.7%)
Nucleolus	Size	large	872/970 cells (89.9%)
		small	98/970 cells (10.1%)
	Shape	round or oval	881/970 cells (90.8%)
		irregular	89/970 cells (9.2%)

normally show uniform chromatin patterns and uniform thickness of nuclear borders. This is a very important difference between cancer cells and non malignant cells.

Large cell carcinoma (Table IV). Large cell carcinoma means large cell, undifferentiated carcinoma. Cellular arrangement in cell-groups of large cell carcinoma is always irregular and the cellular rim is usually poorly defined or not stained. The shape of nuclei is round or oval in 60.7 per cent of 1,234 cells and irregular in 39.3 per cent. Nuclear borders of most cells are very thin (97.5% of 1,234 cells) and the size of nucleus is not uniform. Chromatin patterns of large cell carcinoma cells show fine reticular and/or fine granular in 75.2 per cent of 1,234 cells, coarse reticular in 10.1 per cent and coarse granular in 14.7 per cent.



Figs 5 6 Very fresh adenocarcinoma cells obtained by the TV-brushing technique Fresh nuclei show compact but fine reticular and/or fine granular chromatin patterns Nuclei are round in shape and fully inflated like balloons Nucleoli are round in shape, large in size, and mostly situated near the center of the nucleus The nuclear borders are always very thin. × 2,640

Therefore, precise studies were made to clarify the characteristic morphologic findings of very fresh cancer cells, especially adenocarcinoma cells, obtained by the TV-brushing technique

The morphologic findings of malignant cells and non-malignant cells obtained by the TV brushing technique are summarized in Table V

Tamura[11] of our Center has reported the degeneration process of adenocarcinoma cells of the stomach as follows Fresh cancer cells have fresh nuclei with compact but fine chromatin, and their nuclear borders are very thin In the next stage nuclei of the same cells begin to degenerate and have loosely arranged coarse chromatin but their nuclear borders are still thin Next, nuclei of these cells become somewhat smaller and denser Nuclei enlarge again to show cloudy swelling and then become homogenous

We also observed by phase contrast microscopy that adenocarcinoma cells directly smeared from surgically resected small peripheral lung cancer show the same degenerative process as adenocarcinoma cells of the stomach. From comparative studies of same adenocarcinoma cell-groups using phase contrast microscopy and ordinary light microscopy it was clarified that fresh adenocarcinoma cells generally show fine reticular and or fine granular chromatin patterns, while degenerating adenocarcinoma cells show coarse reticular or coarse granular chromatin patterns. These various chromatin patterns of tumor cells are usually observed in the histologic section of surgically resected adenocarcinoma of the lung where no necrosis is found

Fresh adenocarcinoma cells obtained by the TV-brushing technique mostly showed compact but very fine chromatin pattern of fresh nuclei Nuclei were round in shape and fully inflated like balloons Nucleoli were round in shape large in size, and mostly situated near the center of the nucleus The nuclear borders were always very thin As shown in Figure 2 and described in the Results, even in fresh adenocarcinoma cell-groups obtained by the TV-brushing technique,

TABLE IV *Morphologic findings of large cell carcinoma cells obtained by the TV-brushing technique from peripheral cancer less than 3 cm in diameter (1,234 cells in 10 cases)*

Cellular arrangement in cell groups found on smear		acinous arrangement	0 case
		irregular arrangement	10 cases
Cellular rim		well defined	65/1,234 cells (5.3%)
		poorly defined	575/1,234 cells (46.6%)
		not stained	594/1,234 cells (48.1%)
Shape of nucleus		round or oval	749/1,234 cells (60.7%)
		irregular	485/1,234 cells (39.3%)
Chromatin pattern		fine reticular and/or fine granular	929 cells (75.3%)
		coarse reticular	125 cells (10.1%)
		coarse granular	180 cells (14.7%)
Nuclear border		thin	1,204 cells (97.5%)
		thick	30 cells (2.5%)
Nucleolus	Size	large	78/153 cells (50.9%)
		small	75/153 cells (49.1%)
	Shape	round or oval	128/153 cells (83.6%)
		irregular	25/153 cells (16.4%)

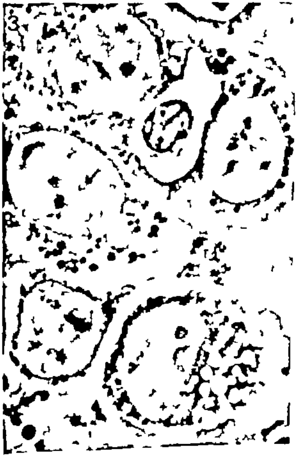


FIG 3 Fresh adenocarcinoma cells obtained by the TV-brushing technique Phase contrast microscopy × [illegible],650.

FIG 4 The same cells are those shown in Fig 3 Fresh adenocarcinoma cells show fine reticular and/or fine granular chromatin patterns Nuclear borders are very thin Papanicolaou stain, × 2,640.

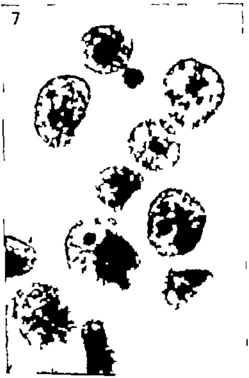


Fig 7 Fresh adenocarcinoma cells obtained by the TV-brushing technique As shown in this figure and Fig 2, even in fresh adenocarcinoma cell groups, various patterns of slight cellular degeneration can be observed This is very importan difference from the finding of non-malignant cells × 2,637

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TABLE V *Morphologic findings of malignant cells and non-malignant cells obtained by the TV-brushing technique from peripheral region of the lung*

	Adenocarcinoma cells	Large cell carcinoma and epidermoid carcinoma cells	Non malignant cells
Cellular arrangement	acinous structure and irregular arrangement of cells are seen in cell groups or cell-clumps	irregular arrangement of cells mostly single cells	*regular arrangement of* cells identified to epithelial layer
Cytoplasm	stained pale blue cytoplasmic rim is frequently poorly defined or not stained	stained faintly so that cytoplasm can rarely be seen	stained well and cytoplasmic rim is distinctly observed sometimes brush border is visible
N/C ratio	increased	markedly increased	not increased
Shape of nucleus	round or oval marked anisokaryosis	polygonal and irregular shape is predominant marked anisokaryosis	round or oval small in size and no anisokaryosis
Nuclear border	mostly thin	thin in very fresh nucleus sometimes not uniform in thickness	uniform in thickness
Pattern of chromatin	mostly fine reticular and/or fine granular compact a few cells having coarse reticular chromatin are mixed	fine reticular and/or fine granular compact but many cells having coarse reticular or coarse granular chromatin are mixed	fine reticular and/or fine granular but chromatin net is not so compact
Nucleolus	large and round situated near the center of nucleus appears like a ball	large or small polygonal or round	small in size and varied in shape appears very flat

various patterns of slight cellular degeneration could be observed. This is a very important difference from the finding of non-malignant cells. Therefore, when the chromatin pattern appears fine reticular and difficulty is experienced in discriminating whether it is a cancer cell or not, it is always necessary to make the observation at x1 000 or more under oil immersion and to note the aforementioned chromatin patterns of various degenerative stages in the same cell-group.

Another special finding is the faintness of the cellular rim. It is presumed that the cancer cell per se carries a negative charge, and therefore the cytoplasm is not stained by anionic dye. Another reason for this faintness is that adenocarcinoma cells have a broad blister consisting of thin hyaloplasm which is not stained with anionic dye, as described by Tamura[11] In the heretofore light microscopy observations, adenocarcinoma cells having cilia could not be observed.

As shown in Figure 1 the TV-brushing technique was found to be effective for all bronchial ramifications. However even by this technique about 17 per cent of peripheral lung cancers could not be diagnosed. When the TV-brushing technique was negative, needle biopsy and aspiration technique from the outside was occasionally effective. We use a very thin needle (0 8 mm inner diameter) made in Japan, and the cellular findings of cancer cells obtained with this needle are almost the same as those by the TV-brushing technique.

Summary

During the past eight years, the TV-brushing technique has been used on 436 cases of peripheral lung lesions, especially small lesions suggestive of lung cancers Out of the 208 confirmed cases of primary lung cancer 172 cases were found to be cytologically positive by this technique for a positive rate of 82.7 per cent.

Considerable differences in morphologic findings were observed between fresh cancer cells obtained by the TV brushing technique and exfoliated cancer cells in the sputum. With precise studies using cancer cells obtained by the TV-brushing technique, the characteristic morphologic findings of very fresh cancer cells in the lung periphery were established.

TABLE I. *Examination Results in Proficiency Testing in Exfoliative Cytology*
July 1969 to June 1970

Score	Number of Errors	Number of Cytotechnologists			
		Test 1	Test 2	** Test	
		PASSED			
100	None	50 (35%)		2	
90	10-point	45 (27 %)	8		
80	10-point	50 (35%)	7	—	
		43 (85%)	6	3	Total
		FAILED			
70	3 10-point	12	1	—	
60	10-point			—	
50	5 10-point	2	—	—	
40	10-point	1	—	—	
30	10-point	0	—	—	
20	10-point	2	—	—	
		25		—	Total

* 3 technicians not retested
** technicians not retested

TABLE II. *Distribution of Cytotechnologists in Permit Laboratories*
July 1969 to June 1970

Number of Laboratories	Number of Cytotechnologists	Pass	Fail
89	0		—
97	168	143	25
Total 186	168	85%	15%

TABLE III. *Results in Proficiency Testing Program of Cytotechnologists in 97 Laboratories*
July 1969 to June 1970

Number of Laboratories	Number of Cytotechnologists	Pass	Fail
79	99	99	0
8	9	0	9
10	60	44	16

uniform and independent interpretation by three pathologists, either in our department or in reference laboratories. Varying usage in terminology for reporting specimens necessitated the selection of three distinct predefined categories of cells to be identified, i [illegible] positive indicating malignancy, negative indicating no malignancy, and an inconclusive or suspicious type. Rating of the five specimens was based on 20 points per smear. A passing grade of 80 points allowed one full error or two one-half errors.

The accuracy of the cytotechnologists in the evaluation of smears was then examined in relation to data recorded from a personnel questionnaire. All cytotechnologists with less than one year of experience performed satisfactorily, whereas those with more than five years, and one to five years experience, had a 20 and 13 per cent failure rate respectively (Table V).

As shown in Table VI most of the screeners were listed in the category "cytotechnologist" and this group also included the greatest number passing and failing. The next largest was supervisors, of whom eight or 28 per cent failed to achieve 80 points. One might surmise that the "supervisor" category may have been erroneously interpreted, were it not for the fact that these cytoscreeners had indicated more than five years of experience, and had reached the supervisor level.

In Table VII, it is seen that attendance at an approved school of cytotechnology bore little rela-

TABLE IV. *Distribution of Failing Cytotechnologists in 8 Laboratories*
July 1969–June 1970

Number of Laboratories	Number of Cytotechnologists Employed in Each Laboratory	Total Number of Cytotechnologists Employed	Pass	Fail
	1		(0)	1 (7)
	2		(0)	2 (2)
	2	8	1 ( )	( )
2			(8)	1 (2)
2	9	18	(14)	2 (4)
			8 (8)	8 (5)
	5	5	1 (14)	1 (1)
Total 18			(44)	(25)

TABLE V. *Experience of Cytotechnologists*

Number of Years	Number of Cytotechnologists	Pass	Per cent	Fail	Per cent
Less than 1 year	19	19	100	0	0
1 to 5 years	76	66	77	10	13
More than 5 years	73	58	80	15	20
Total	168	143		25	

TABLE VI. *Position of Cytotechnologists*

Category	Number of Cytotechnologists	Pass	Per cent	Fail	Per cent
Supervisor	29	21	72	8	28
Cytotechnologist	126	11	89	14	11
Teaching	2	2	100	0	0
Other	11	8	73	3	27
Total	168	143		25	

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New York State Computerized Proficiency Testing Program in Exfoliative Cytology Evaluation

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Proficiency testing or external quality control is only one method by which an external agency—in our case the State—may evaluate the performance of a clinical laboratory In order to appraise the overall capability and reliability of a laboratory an effective program should determine several other elements. These include the education and experience of personnel, record keeping, equipment, facilities and continuous assessment of appropriate quality control methods.

The ultimate purpose of proficiency testing is to protect individual and public health by establishing acceptable reproducible standards of performance in all laboratory disciplines. The evaluating agency may then formulate guidelines and realistic objectives on which to base regulatory action.

On this basis, a proficiency testing program in exfoliative cytology was established in New York State in 1968[3] as a result of the enactment of Article 5, Title V of the New York State Public Health Law A computerized program was designed to test and record the performance of cytotechnologists and tabulate their qualifications, and experience. In addition, a survey of laboratories by questionnaires and on-site inspection by clinical laboratory investigators permitted the accumulation of a variety of data. Many difficulties, some unforeseen, were encountered in designing a quality control or proficiency testing program. Perhaps of greatest importance was the realization that optimum evaluation lay in the establishment of external as well as internal quality control standards. However initially it was felt that limitation to an external testing program appeared to be the most rapid and direct method of assessing the diverse types of cytology laboratories. Variability in personal interpretation as well as accuracy and reproducibility of results are an integral part of cytodiagnosis, and methods to evaluate and possibly eliminate these inherent qualities present a major consideration in a well-formulated testing program.

Our experience and results of the first complete year are presented, as well as recommendations which may serve as guidelines for this and future programs in the quality control of exfoliative cytology

RESULTS

Table I illustrates the performance rating of 168 cytotechnologists who were tested during the first year of the program. Test 1 shows that 143 or 85 per cent achieved a satisfactory passing rate of 80 points, and 25 or 15 per cent failed. Of interest is the same pass/fail rate recorded during the initial six month period of the program, wherein testing was only tabulated on 111 cytotechnologists.[1] On Test 2, 16 cytotechnologists who had failed the first test were successful, four were not, and five were unavailable for retesting On a third test, three of the four failing on the second test now passed, and one was not retested. Hence, a total of 162 or 96 per cent of the cytotechnologists performed satisfactorily in the 1969–1970 program.

Table II shows the distribution and performance of the 168 cytotechnologists in the 186 laboratories with permits in cytology Ninety-seven laboratories employed cytotechnologists, while in 89 laboratories all screening was performed by the cytopathologist.

Table III shows that in 79 laboratories there were no failing cytotechnologists while Table IV shows the random distribution of the 25 cytotechnologists in 18 laboratories who failed on the first test.

The high rate of performance may be due to either a facile test and lenient scoring, or highly competent cytotechnologists. The number of specimens was limited to three cervical-vaginal and two sputum smears selected on the basis of

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TABLE Examination Results Proficiency Testing Exfoliative Cytology
July 1969 to June 1970

ore	Number of Errors	Number of Cytotechnologists			
		Test	Test 2	** Test 3	
		PASSED			
00	None	39 (35%)			
90	1 10-point	45 (37%)	5		
80	10-point	59 (25%)	7	—	
		143 (85%)	1		Total
		FAILED			
70	10-point		1	—	
60	10-point	8	3	—	
50	10-point	2	—	—	
40	4 10-point		—	—	
30	10-point	6	—	—	
20	8 10-point	2	—	—	
		25		—	Total

5 technicians not retested
** technicians not retested

TABLE II. Distribution of Cytotechnologists in Permit Laboratories
July 1969 to June 1970

	Number of Laboratories	Number of Cytotechnologists	Pass	Fail
	89	0	—	—
	97	168	143	25
Total	186	168	85%	15%

TABLE III Results in Proficiency Testing Program of Cytotechnologists in 97 Laboratories
July 1969 to June 1970

Number of Laboratories	Number of Cytotechnologists	Pass	Fail
79	99	99	0
8	9	0	9
10	60	44	16

uniform and independent interpretation by three pathologists, either in our department or in reference laboratories. Varying usage in terminology for reporting specimens necessitated the selection of three distinct predefined categories of cells to be identified, positive indicating malignancy, negative indicating no malignancy and an inconclusive or suspicious type. Rating of the five specimens was based on 20 points per smear; passing grade of 80 points allowed one full error or two one-half errors.

The accuracy of the cytotechnologist in the evaluation of smears was then examined in relation to data recorded from personnel questionnaire. All cytotechnologists with less than one year of experience performed satisfactorily whereas those with more than five years, and one to five years experience, had 20 and 13 per cent failure rate respectively (Table V).

As shown in Table VI most of the screeners were listed in the category "cytotechnologist" and this group also included the greatest number passing and failing. The next largest was supervisors, of whom eight or 28 per cent failed to achieve 80 points. One might surmise that the "supervisor" category may have been erroneously interpreted, were it not for the fact that these cytoscreeners had indicated more than five years of experience, and had reached the supervisor level.

In Table VII, it is seen that attendance at an approved school of cytotechnology bore little rela-

TABLE IV Distribution of Failing Cytotechnologists in Laboratories
July 1969 to June 1970

Number of Laboratories	Number of Cytotechnologists Employed in Each Laboratory	Total Number of Cytotechnologists Employed	Pass	Fail
			0 (0)	7)
1		2	0 (0)	2 (2)
4	2		( )	( )
			3 (6)	1 (2)
2	9	18	(14	2 (4)
1			(6)	(5)
			1 (14)	1 (
Total 18			(44)	(25)

TABLE V Experience of Cytotechnologist

Number of Years	Number of Cytotechnologists	Pass	Per cent	Fail	Per cent
Less than 1 year	19	19	100	0	0
1 to 5 years	76	66	77	10	13
More than 5 years	73	58	80	15	20
Total	168	143		25	

TABLE VI Position of Cytotechnologists

Category	Number of Cytotechnologists	Pass	Per cent	Fail	Per cent
Supervisor	29	21	72	8	28
Cytotechnologist	126	112	89	14	11
Teaching	2	2	100	0	0
Other	11	8	73	3	27
Total	168	143		25	

TABLE VII *Training of Cytotechnologists*

Training	Number	Per cent	Pass	Per cent	Fail	Per cent
School of Cytotechnology	106	63	92	86	14	14
Other	62	37	51	82	11	18
Total	168		143		25	

TABLE VIII *Professional Status of Cytotechnologists*

Registered	Number	Per cent	Pass	Per cent	Fail	Per cent
Cytotechnologist	72	43	62	86	10	14
Not	96	57	81	85	15	15
Total	186		143		23	

TABLE IX *Attendance and Membership Distribution*

Attend Cytology Meetings		Attend Meetings (Other)		ASC Member	
Yes	No	Yes	No	Yes	No
78	90	49	119	48	120

tion to performance, since there was no significant difference in the performance of those with dissimilar training

Table VIII indicates the professional status of cytotechnologists as demonstrated by formal registration Ninety-six non-registered cytotechnologists were compared to the 72 who were registered Apparently registered cytotechnologists, of whom the majority were required to attend an approved school of cytotechnology did not exhibit a higher rate of proficiency on this test

Several questions relating to length of employment in present position, full or part-time employment, attendance at cytology and or other meetings, and membership in the American Society of Cytology completed the personnel questionnaire. Replies were of interest mainly in tabulating information concerning job stability and working interests of cytotechnologists rather than relevance to performance (Table IX)

Information about specific educational background as related to holders of other registration categories and degrees was incomplete, since this question was answered by only 123 of the 168 cytotechnologists. It is possible that the numerous variables in the educational background of cytotechnologists precluded exact classification to fit the listed categories (Table X)

The Cytology Laboratory

A survey of all laboratories engaged in diagnostic cytology was evaluated from the data supplied in the laboratory questionnaire.[3] Data were recorded on all 186 laboratories however detailed analysis was limited to the 97 which employed the 168 cytotechnologists (Tables II and III) Analysis of this information which might have a bearing on cytotechnologist performance was then attempted.

Tabulation was made of the type of laboratory (hospital, independent or other) volume and type of specimens, number of pathologists, frequency of visits and instruction by the pathologist. Internal quality control as represented by the percentage review by the pathologists of negative and positive specimens, and availability of follow up data, were examined No significant disparities were evident in these 18 laboratories as compared to the 89 laboratories which employed cytotechnologists who performed satisfactorily

Discussion

The primary object of quality performance is the need for precision, accuracy and reproducibility In contrast to other laboratory disciplines, where machine determinations permit the use of Gaussian curves, coefficients of variation and standard deviations, such conventional guideposts of quality control are not applicable to cytodiagnosis Reliance has to be placed on human factors, wherein knowledge of the discipline, education and experience assume the major role. Specimen quality must include an adequate representative test sample which is well preserved and stained, however these factors in themselves are valueless if not subjected to competent evaluation by the individual screener Thus it becomes essential that a proficiency test in cytodiagnosis must be primarily "person" oriented.

Our state proficiency testing program found certain relationships among the performance of

TABLE X *Education of Cytotechnologists*

Degrees	Number	Pass	Fail
HT MT (ASCP)	10	7	3
BA/BS MA/MS	57	48	9
Other	56	48	8
No answer	45	40	5
Total	168	143	25

cytotechnologists which appeared to contradict some of the conclusions reached from studies based on the certification examination. There did not appear to be any appreciable difference between the performance of cytotechnologists who had received their training in laboratories accredited as schools of cytotechnology by the American Medical Association's Council on Medical Education, and those who had been trained in laboratories not so recognized. We would agree with their conclusion however that cytotechnologists with many years of experience did not perform as well as the more recent graduates, who attended accredited schools of cytotechnology.

Initial testing in our program was basically designed to explore all laboratories, general hospitals as well as independent laboratories, many of which confine their examinations predominantly to material from the reproductive and/or respiratory systems. Therefore, our limited testing, omitting smears from all body fluids, in effect excluded comparison with the broader practical evaluation of the certification examination. Our experience to date suggests that increasing the number of specimens to ten smears as well as including pertinent information such as last menstrual period and age of patient may permit greater diversity in scoring and better assessment of errors in cytotechnologist performance. Our newly devised test form also includes clarification of report categories with reference to cytohistologic correlations, so that more exact identification of pathologic lesions may be identified.

Internal quality control methods as practiced in laboratories presented the most difficult problem to evaluate. Whereas many data in our questionnaire related to the *modus operandi*, it appeared an almost impossible task to assign significant weight to or quantify practices such as the percentage of negative or other categories reviewed by the pathologist. The federal Clinical Laboratory Improvement Act of 1967[3] outlines certain minimal specifications wherein licensed cytology laboratories engaged in interstate commerce require rescreening by a pathologist or qualified supervisor of 10 per cent of cervical vaginal specimens diagnosed as negative, in addition to all specimens other than those from the cervical-vaginal tract. Our forms have now been revised to concentrate on these and similar aspects where necessary supervision is considered essential.

So far little has been said about accuracy and reproducibility of results in the laboratory. Unconscious bias by the cytoscreener in internal quality control may be decreased by the constant review of "blind" smears carefully introduced into daily screening. Records of the cytoscreener's accuracy should be assiduously kept to determine the efficiency rate of the individual. Cytoscreeners differ in their degree of concordance with one another and the magnitude of this difference is, on the average, somewhat greater for screeners in different centers than for screeners in the same center. Cognizance of this important aspect of the individual accuracy rate prompted incorporation of questions which might determine the relevancy of this observation in our revised form.

Discussion so far has centered around the cytotechnologist and the necessity for intralaboratory quality control. Traditionally, cytology laboratories include at least two professional levels who are responsible for screening and interpretation of specimens. In laboratories with a small volume, the pathologist or cytopathologist often screens and interprets the smears. Laboratories with a greater workload, usually employ screeners who perform primary scanning of smears and note existing abnormalities. These abnormal findings are then referred to the pathologist for final interpretation or diagnosis. Frequently a supervisory cytotechnologist who has had an arbitrary time span of experience is interposed between the screener and the pathologist. Since, as noted in Table II, 89 of the 186 laboratories employed no cytotechnologists, the question arises, how can these laboratories be assessed? Should performance as an indicator of quality control be extended to include all who screen and practice cytodiagnosis? An evaluation of acceptable quality control in exfoliative cytology will never be valid or reflect the true state of cytodiagnosis unless all laboratories submit to the same standards. If the answer is in the affirmative to test all professional levels, would a cytopathologist evaluation program fill this present void? Again, will the implementation of automated cell analysis introduce yet another facet in the problem of proficiency evaluation. Attention may then have to be paid to methods of processing specimens as it is now in effect in clinical chemistry, hematology and other laboratory disciplines. The difficulties experienced in evaluating laboratories which employ acridine orange techniques as an alternate to the Papanicolaou stain, should remind one that advocates for different methods cannot be ignored. After more than two years of experience with this state proficiency testing program, certain recommendations and suggestions may be made.

1. Additional instruction or refresher courses should be made available to cytotechnologists who fail to perform satisfactorily after three tests in a year.

2. Voluntary retirement or transfer to another discipline for "misplaced" cytoscreener should be effected.

3. The State should make available or arrange for on-going instruction, whether by the institution of workshops, seminars, or conferences. A Kodachrome-of-the-Month teaching series has been

mailed to all cytology laboratories since the onset of our proficiency testing program, which is now in its third year

4 Special attention should be given to the upgrading of laboratories where insufficient or deficient *internal quality control exists or to* where outmoded or unsatisfactory methods and procedures are employed

5 There should be review and comparison of internal and external standards after what appears to be a suitable interval for this evaluation.

6 The promotion of constant high levels of performance in cytodiagnosis should be the future goal in this State as exemplified by mutual cooperation with all cytology laboratories, without the ghost of censure stimulating overt incentive.

Summary

An external quality control or proficiency testing *program in exfoliative cytology was developed in* New York State. The program resulted from the enactment of the Clinical Laboratory Services Act of 1964 and it provided data on 168 cytotechnologists and *186* laboratories from *July 1 1969* through June 30, 1970

While the sample of laboratories, cytotechnologists, and test specimens appears too small for statistical analysis, the data suggest certain implications which should be re-evaluated for accuracy and replication. These are

1 Education and length of service appear to have little relevance in assessing proficiency

2 Cytotechnologists certified by a national agency when compared to non-certified individuals who have had less formal training, do not necessarily perform with greater efficiency

3 Membership in specific scientific organizations bears no relation to performance.

4 Hierarchical position in the laboratory does not guarantee better performance.

5 Postgraduate educational efforts by cytotechnologists in this state are not particularly apparent and have no relevancy in assessing proficiency

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Calcified Bodies and the Intrauterine Device

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Reports on incrustation on the intrauterine device have recently appeared in the literature. These deposits were found in loops from women who had been wearing them for 15 to 40 months many of whom had intractable vaginal bleeding. The deposits tended to be firm and rough and on chemical analysis were composed largely of protein, calcium and magnesium. These reports do not include cytology or histology and give no information as to whether the deposits are capable of provoking foreign body reaction.

The patient described below had been using Saf-T-Coil for 18 months. She presented with menorrhagia and her cervical smear contained numerous calcified bodies and foreign body giant cells.

CASE REPORT

Mrs N 31 year old woman, gravid 2, para 2, was first seen in September 1969 complaining of painful, heavy prolonged periods and premenstrual tension. A Saf-T-Coil had been in situ for 18 months. On gynecologic examination there was no significant abnormality. The coil was removed and the patient given an oral contraceptive. She was seen again in October 1970 having stopped taking the contraceptive in August hoping to become pregnant. She was asymptomatic and gynecologic examination was normal.

Cytological Findings

A cervical smear was taken in September 1969. It is not known whether the smear was taken before or after removal of the coil. It showed the following features. The squamous epithelium was within normal limits and there were many sheets of endometrial-like cells. A number of lilac bodies were present with an irregularly ovoid or round shape and an internal structure of round, oval or irregular concentric rings. Some of the larger forms had the appearance of multiples of the smaller bodies. Approximately 90 of these bodies could be seen in the smear the majority contained within multinucleate histiocytes (Figs 1 and 2). Most of these bodies stained deep lilac. A few of the smaller ones were paler and stained less intensely. Large numbers of histiocytes were present and approximately 90 large multinucleate forms. The majority of these were of foreign body type with an irregular arrangement of their nuclei throughout the cytoplasm. In few the nuclei were arranged peripherally. The follow-up smear in October 1970 taken 13 months after removal of the intrauterine device was completely normal.

DISCUSSION

There is no histology available for this patient. The origin and nature of these foreign bodies must, therefore, be matter of speculation. Although many of these bodies were ovoid rather than round, number closely resembled psammoma bodies seen in ovarian and other lesions. However these structures had clearly provoked foreign body reaction and when intracellular were related largely to foreign body giant cells and not to epithelial cells of the type reported by Picoff and Meeker in their case with benign papillary ovarian lesion. The abundance of these bodies would support an intra rather than extra uterine origin. The possibility of calcified ova, e.g. enterobius vermicularis or Schistosoma haematobium is unlikely in view of their abundance and variation in size and appearance. It is felt that the probable diagnosis in this patient with an intrauterine device in situ for 18 months and history of heavy periods is a foreign body reaction provoked by incrustation on and around the device. This view is supported by the completely normal follow-up smear 13 months after removal of the device.

Sagiroglu and Sagiroglu examined loopal smears from 52 women wearing the Lippes loop from 3 hours to 39 months. They found mononuclear macrophages in abundance. These macrophages increased continuously in proportion to the duration of insertion. Foreign-body giant cells were numerous in the loopal smears from patients who had used the IUD' for longer than 3 months. Both the mono and multinucleate histiocytes showed phagocytic cytoplasmic inclusions. These included lightly colored cyanophilic inclusions

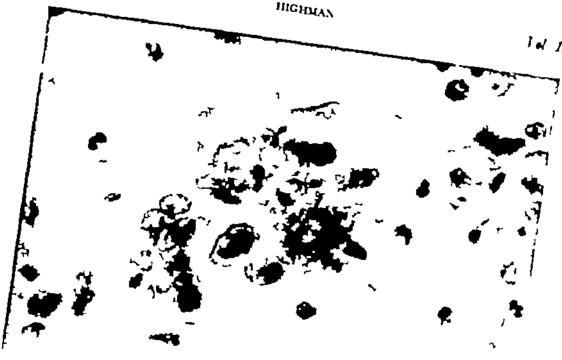

FIG 1 Cervical Smear Small calcified body within multinucleate histiocyte (×200)

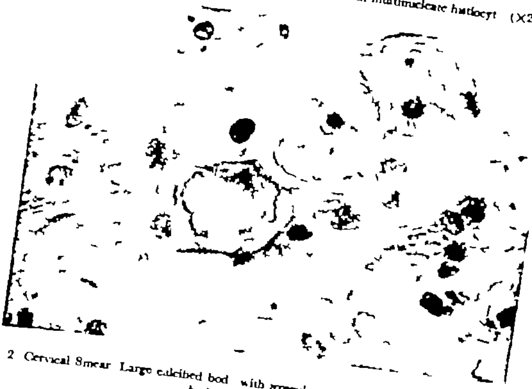

FIG 2 Cervical Smear Large calcified body with irregular concentric ring structure surrounded by histiocytes (×200)

which could not be identified and a large polyhedral body surrounded by numerous macrophages This was probably thought to be an ovum or blastocyst Both the unidentified cyanophilic inclusions and the large polyhedral body resemble the deposits in the smear reported above and may have the same origin Ishihama, *et al* reported on vaginal smears from 1 038 women 75 per cent of whom had worn the IUD for 5 or more years. He found the incidence of foreign-body giant cells in these smears extremely low

There are several reports on the histology of curettings in IUD users. These indicate a variety of slight acute, subacute and chronic inflammatory reactions with the polyethylene device. These lesions are usual focal being maximal at the points of contact. In few instances squamous metaplasia of the surface endometrium is seen. The cellular infiltrate appears to consist of varying proportions of neutrophils, plasma cells and mononuclears. These mononuclears are difficult to precisely identify in hematoxylin-stained sections. Calcified bodies and foreign body giant cells are not described.

The discrepancy between the cytologic features in cervical, vaginal and loopal smears, and the endometrial histology has been suggested by Sagiroglu to be due to proliferation of macrophages in the endometrial cavity rather than endometrial tissue. This situation would be analogous to that in lung secretions, pleural and peritoneal fluids and breast cyst fluids. In these the large numbers of macrophages which, presumably originate in the parent tissue are perpetuated and increased by proliferation in the fluid medium in which they appear. Sagiroglu observed mitotic activity in many of the histiocytes in the loopal smears. Occasional mitoses were observed in the histiocytes in the reported smear. The giant histiocytes observed in the loopal smears presumably represent both direct effect of the I.U.D. and reaction to the loopal deposit. In the case reported these features are undoubtedly exaggerated by the large numbers of calcified bodies. There appears to be no explanation for these bodies other than the long term presence of the I.U.D. From the lack of case reports in the literature this would seem to be an uncommon finding. The reason for their occurrence in this case remains obscure.

SUMMARY

Many calcified bodies and foreign body giant cells were found in the cervical smear of a woman with menorrhagia and a Saf T-Coil in situ for eighteen months. These disappeared after removal of the coil and are considered to have been provoked by it.

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The author wishes to thank Mr. D. W. T. Roberts for kind permission to publish this case.

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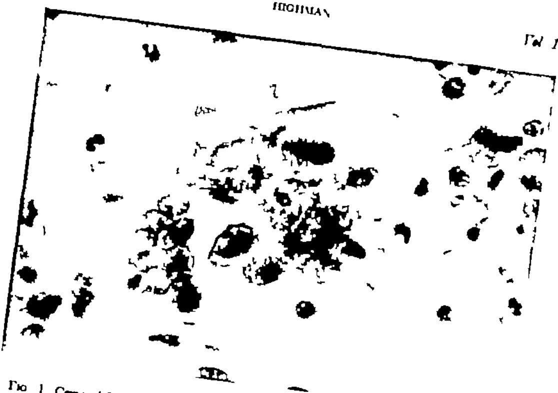

Fig. 1. Cervical Smear. Small calcified body within multinucleated histiocyte. (×200)

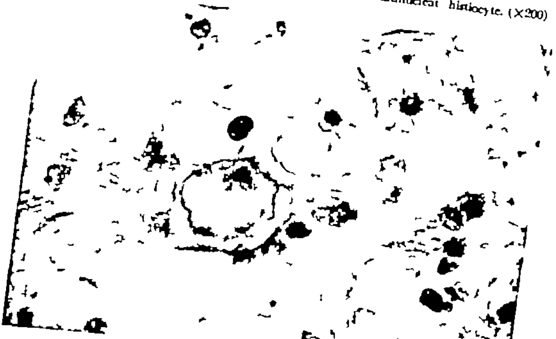

Fig. 2. Cervical Smear. Large calcified body with irregular concentric ring structure surrounded by histiocytes. (×200)

which could not be identified and a large polyhedral body surrounded by numerous macrophages. This was probably thought to be an ovum or blastocyst. Both the unidentified cyanophilic inclusions and the large polyhedral body resemble the deposits in the smear reported above and may have the same origin. Ishihama, et al. reported on vaginal smears from 1 058 women, 75 per cent of whom had worn the IUD for 5 or more years. He found the incidence of foreign-body giant cells in these smears extremely low.

There are several reports on the histology of curettings in IUD users. These indicate a variety of slight acute, subacute and chronic inflammatory reactions with the polyethylene device. These lesions are usually focal being maximal at the points of contact. In a few instances squamous metaplasia of the surface endometrium is seen. The cellular infiltrate appears to consist of varying proportions of neutrophils, plasma cells and mononuclears. These mononuclears are difficult to precisely identify in hematoxylin-stained sections. Calcified bodies and foreign body giant cells are not described.

The discrepancy between the cytologic features in cervical, vaginal and loopal smears, and the endometrial histology has been suggested by Sagiroglu to be due to proliferation of macrophages in the endometrial cavity rather than endometrial tissue. This situation would be analogous to that in lung secretions, pleural and peritoneal fluids and breast cyst fluids. In these the large numbers of macrophages which, presumably originate in the parent tissue are perpetuated and increased by proliferation in the fluid medium in which they appear. Sagiroglu observed mitotic activity in many of the histiocytes in the loopal smears. Occasional mitoses were observed in the histiocytes in the reported smear. The giant histiocytes observed in the loopal smears presumably represent both a direct effect of the IUD and a reaction to the loopal deposit. In the case reported these features are undoubtedly exaggerated by the large numbers of calcified bodies. There appears to be no explanation for these bodies other than the long term presence of the IUD. From the lack of case reports in the literature this would seem to be an uncommon finding. The reason for their occurrence in this case remains obscure.

SUMMARY

Many calcified bodies and foreign body giant cells were found in the cervical smear of a woman with menorrhagia and a Saf T-Coil in situ for eighteen months. These disappeared after removal of the coil and are considered to have been provoked by it.

ACKNOWLEDGMENT

The author wishes to thank Mr. D. W. T. Roberts for kind permission to publish this case.

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Findings of Microfilarial Larval Stages in Gynecologic Smears

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There is no reference in medical literature to the presence of larval stages with microfilarial characteristics in the gynecologic smears. The present study was undertaken after the observation of these parasites in some smears, which were routinely examined in the Instituto de Anatomía Patológica, Hospital Vargas, Caracas, Venezuela. We were able to identify these microfilariae as belonging to the species of *Onchocerca volvulus*. It is most probable that this type of filaria was brought to America by Negro slaves and was described for the first time in Guatemala by Robles in 1915. In Mexico the infectious foci were found in the states of Chiapas and Oaxaca.

In Venezuela, the first case of Onchocercosis was described by Potenza *et al.* in 1949, during the histologic study of nodules of patients from the eastern region of the country. Other workers[1-4] have done epidemiologic studies and have demonstrated the existence of endemic foci of Onchocercosis in the central and northeastern zones of the country. At present, it can be stated that this disease has a geographic distribution with predominance in the Cordillera de la Costa.

MATERIALS AND METHODS

This study was carried out on 15,261 patients who came from various parts of the country and were residing in Caracas, their ages ranging from 26 to 58 years old. With one exception where genital tuberculosis was suspected, all other cases were screened for gynecologic cancer. Seven patients were in menopause. Exocervical, endocervical and endometrial smears were examined. Cervical smears were done with the wooden spatula and endocervical material was obtained with a cotton swab. Endometrial smears were taken only from the seven menopausal women, who presented metrorrhagia.

RESULTS

The presence of parasitic larvae with microfilaria characteristics was observed in 12 cases. Smears that contained these microfilariae showed no alterations, as compared to the rest of the non-neoplastic material. The smears consisted predominantly of polymorphonuclear neutrophils and histiocytes and all of them presented intact and partially destroyed red blood cells, some well preserved epithelial cells and cell debris were also found. In some preparations, especially from young women, epithelial cells from deeper layer were seen, which suggested the presence of erosive zones with epithelial detachment (Fig. 1). No cancerous cells were found in the smears with proven microfilaria. With the exception of one case, whose endometrial smear showed scarce, atypical cells, possibly cancerous, all other smears having microfilariae were reported as inflammatory.

In some smears, microfilariae were numerous and in others, scarce. Most of them appeared fragmented and some were thick and shortened (Fig. 2). Due to these structural alterations and retraction, and to the scarcity of material, it was difficult to classify the parasites.

In the few fairly well preserved specimens, the following morphologic characteristics were observed (Fig. 3): the rounded, anterior end had no visible probes, the posterior end had no nucleus and there was absence of a sheath. The cuticle was transparent, without transverse striation. Underneath the cuticle some subcuticular cells, intensely stained, were observed. In some specimens, spaces corresponding to those of the nervous rings could be noted, and were placed at about one-fifth the length of the microfilaria, starting from the anterior end. A protrusion situated at about one-third the length of the larva was observed once and was referred to as the excretion pore. In other two specimens, an anal pore, situated towards the posterior end was observed. Yet in another specimen, a clear distinguishable and presumable germinative cell was noted. It was situated near the anterior part of the anal pore. The so-called internal body (reserve material) was seen in some specimens. It occupied almost 70 per cent of the parasitic body. The size of the larvae, measured in 10 specimens, ranged from 226 to 330 μ.

DISCUSSION

The results herein reported show the presence of certain parasitic larvae in the vaginal medium.

Fig. 1. Gynecologic smears showing larva surrounded by cells from the deeper layers of the cervical mucosa. ×850

This habitat is not appropriate for their survival and neither are fixation and the Papanicolaou stain adequated for microfilariae. These unfavorable conditions probably caused the morphologic alterations observed. The absence of internal structural details made classification rather difficult. Nevertheless, the parasitologists who examined these smears agreed that we were dealing with microfilariae.

In Venezuela, up to this date, the following species of filariae have been found in man: 1) *Wuchereria bancrofti*, of limited geographic distribution. The microfilaria of this species of filaria presents sheath and also specific internal structures. 2) *Mansonella ozzardi* and *Dipetalonema perstans* until now found only in the natives of the Orinoco basin. The microfilaria found in the gynecologic smears does not have characteristics of the above species and furthermore it was found in persons who did not come from this endemic zone. 3) *Onchocerca volvulus*, the most abundant filaria in the country. At present it has been found in the north-central and eastern states. This geographic distribution and the morphologic characteristics shown by the parasites observed in the smears, permitted identification of these parasites as larval stages of *O. volvulus*.

It is difficult to explain the presence of this type of parasite in the genital tract of persons whose multiple skin biopsies for microfilariae were negative. Perhaps the most suggestive hypothesis is that these larvae were present in the mucosa of the genital tract and due to the ulcerating lesions of the cervix came up to the surface and were scraped out together with the cervical material.

It is not known whether the mechanical action of the parasites in the gynecologic tract and their

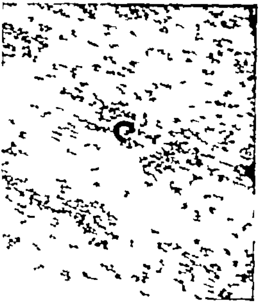

Fig. 2. Inflammatory endocervical smear showing abundant neutrofils, histiocytes and mucus. In the center, fragment of larva which appears thick and retracted. ×340

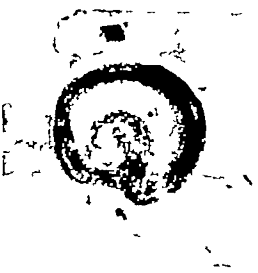

Fig. 3. Microfilaria of *Onchocerca volvulus* present in gynecologic smear. ×1,400

secreted toxins could cause the mucosal lesions (local inflammatory reactions and epithelial ulcerations) observed.

It is well known that microfilariae of *O. volvulus* are absent from the peripheral blood, their passage through the circulatory system being brief and transitory. Therefore, they are found only in dermal tissue. At present, no one has investigated the material obtained from the cervical scarification in the infected patients. Further studies on this matter are needed to clarify the present findings. Patients whose gynecologic smears presented microfilariae, did not show apparent symptoms that could be related to Onchocercosis, such as skin nodules and keratitis. However, it is known that in Venezuela a large number of Onchocercosis cases are asymptomatic[2] and ocular lesions are less frequent than in the cases observed in Guatemala. Moreover, in one of the studied cases, where a thorough investigation for microfilaria on *O. volvulus* was possible, we did not find any parasites. However, the intradermal reaction to the *Filaria gutturosa* cattle antigen gave a positive result.

The present study indicates that in asymptomatic patients from endemic zones of Onchocercosis, microfilariae could be found in the genital tract and these findings could be significant from the pathologic point of view and are worthwhile investigating.

SUMMARY

The presence of microfilariae with *Onchocerca volvulus* morphologic characteristics has been observed in gynecologic smears. Out of 15,261 exocervical, endocervical and endometrial smears, 12 had microfilariae. Using fixative and Papanicolaou staining techniques it was rather difficult to study the structure of the microfilariae. The majority of them showed structural alterations due to an unfavorable vaginal medium and cytologic procedures. It was not possible to study their internal morphology. However, the general characteristics together with the geographic distribution of Onchocercosis in Venezuela, suggested the identification of microfilariae as larvae of *O. volvulus*. The significance of this pathologic finding should be further investigated.

ACKNOWLEDGMENTS

The author wants to express her thanks to Dr. Félix Pifano for the identification of the larvae; to Dr. Renato Olavarría for his interest in the clinical evolution of the patients, and to Drs. Gloria M. Villegas and Fortunato Rosa for their encouragement during the preparation of the manuscript.

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A Cytologic Study of Dipetolenema Perstans in Cervical Smears

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Kampala, Uganda

Dipetolenema Perstans has an extensive distribution in Western and Central Africa, on the East Coast of South America and mildly endemic in Algeria and Tunis.[1] With increasing movement of populations, infection may occur in people other than the indigenous inhabitants of these areas, and we therefore consider it worthwhile to draw attention to the presence of this parasite in cervical smears.

In Uganda nearly all elderly people show this parasite in the blood. The infection seems to produce no symptoms in majority of cases.[2]

MATERIALS AND METHODS

In 40,000 routine cervical smears examined for malignant cells in the exfoliative cytology department of Makerere University Medical School during the period 1966–1970, Dipetolenema perstans were noted in 243 smears. The smears were taken from patients ranging in age from 20 to 60 years. All smears were stained by the Papanicolaou method. The parasites were clearly recognized as unsheathed microfilariae with blunt tail and nuclei up to the tip of the tail (Figs. 1 and 2). They measure approximately 200 microns in length by 4.5 microns in diameter.

CYTOLOGIC FINDINGS IN DIPETOLENEMA PERSTANS POSITIVE CERVICAL SMEARS

The majority of the smears were obtained from patients in the reproductive age group and they had the usual number of superficial and intermediate cells. There were 13 smears taken from the postmenopausal women, showing low oestrin effect, four from pregnant women and ten smears were obtained from patients with clinical cervical carcinoma. The number of the parasites seen in the smears examined varied from a single one to 50 in one smear.

Blood. Blood was present in all the smears. In 21 smears it was the predominant feature and in the remaining smears it varied from few to moderate number of red blood cells.

Inflammatory Cells. All the smears with the exception of ten contained inflammatory cells consisting of polymorphonuclear leukocytes, histiocytes and occasional plasma cells. The inflammatory cells were scattered throughout the smears and were not confined to the immediate vicinity of the parasites.

Bacterial and other Parasites. Bacteria other than Doderlein bacilli were present in 64 smears.

Trichomonas Vaginalis. These were seen in 84 smears and were numerous in 30 smears.

Candida Albicans. Candida Albicans filaments were seen in 36 smears.

Endocervical Cells. These were present in 60 smears and they were numerous in 21 smears.

Atypical Cells. 109 smears of the 243 examined showed atypical cells, of which 84 contained Trichomonas vaginalis. The cellular atypicality included variations in nuclear size, binucleation, nuclear enlargement, nuclear pyknosis and karyorrhexis, marked eosinophilic discoloration of cells of all layers, concentration of nuclear chromatin

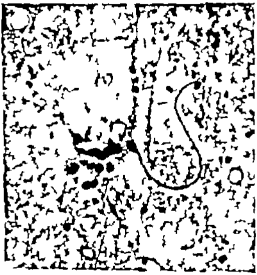

FIG. 1. Dipetolenema perstans in cervical smear (× 100).

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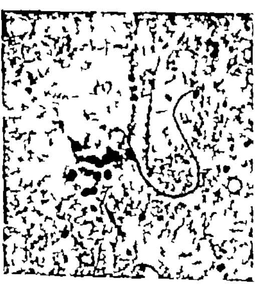

FIG. 1. Dipetolenema perstans in cervical smear (× 100).

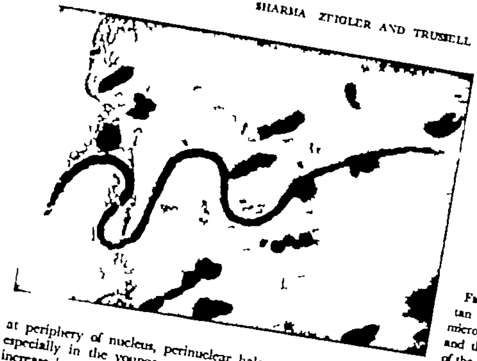

FIG — Dipetolenema perstans identified as unsheathed microfilaria with blunt tail and the nuclei up to the tip of the tail (× 1,000)

at periphery of nucleus, perinuclear haloes and especially in the younger age group a marked increase in the number of parabasal cells. These changes are associated with trichomonas and bacterial infections and are of nonspecific nature. In 25 smears there were clusters of atypical endocervical cells showing nuclear enlargement prominent nucleoli, multinucleation and occasionally bare nuclei, the change suggestive of cervicitis.

Dyskaryotic Cells In five smears, dyskaryotic cells were seen On further investigations two of them were diagnosed as cases of carcinoma in situ of the cervix.

Malignant Cells Malignant cells were seen in 12 smears. Ten of them were cases of clinical carcinoma of the cervix and remaining two were microinvasive carcinoma of the cervix

DISCUSSION AND CONCLUSION

Cytologically there were no constant findings which could be attributed conclusively to Dipetolenema perstans infection The only positive finding in all the smears was the presence of blood which was the predominant feature in few smears and in the remaining it varied from few to moderate number of red blood cells. This undoubtedly is the reason for the high incidence of cervical pathology found in the present series of cases. The amount of blood in the cervical smear had no relation to the number of parasites in the smear The cause of this bleeding in these patients is shown in Table I From this it appears that if the parasites are present in the patient's blood and the cervical smear is taken from such a patient at the time when the patient is bleeding either from the uterus or cervix, one is likely to find these parasites in the smear

TABLE I

Cause of Bleeding	No. of Cases
Menstruating at the time of taking cervical smear	147
Cervical erosion with contact bleeding	66
Bleeding from cervical polyp	3
Postmenopausal bleeding	13
Carcinoma of the cervix	10
Treated threatened abortion	4
	13

No definite pathogenecity has been demonstrated in persons who have this infection, although recently some workers have suggested that this parasite may produce mild symptoms like joint pains and headache in some cases and occasionally may produce serious clinical illness[1-4]. So far this parasite is not known to cause any lesions of the female genital tract, and in this retrospective study we also did not find any pathology which could be attributed to this infection

SUMMARY

In 40 000 cervical smears examined Dipetolenema perstans were seen in 243 smears There were no constant cytologic findings which could be attributed conclusively to this infection In endemic areas most of the indigenous people harbour these parasites in the blood and if the patient is bleeding either from the uterus or cervix at the time of taking cervical smears, one is likely to find these parasites in the smears.

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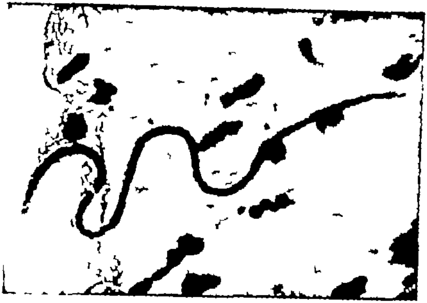

FIG. 2. Dipetalonema perstans identified as unsheathed microfilaria with blunt tail and the nuclei up to the tip of the tail (X 1 000)

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SUMMARY

In 40 000 cervical smears examined Dipetalonema perstans were seen in 243 smears There were no constant cytologic findings which could be attributed conclusively to this infection In endemic areas, most of the indigenous people harbour these parasites in the blood and if the patient is bleeding either from the uterus or cervix at the time of taking cervical smears, one is likely to find these parasites in the smears

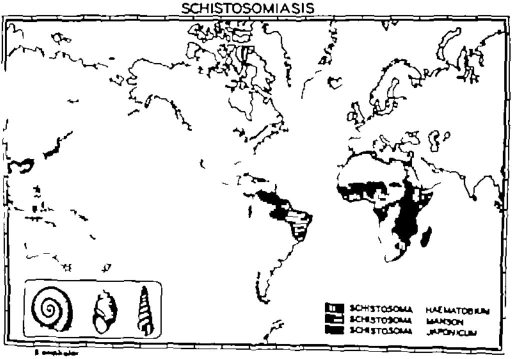

Fig. 1 Map showing distribution of three main species of Schistosome (With acknowledgments to Ash and Spitz.)

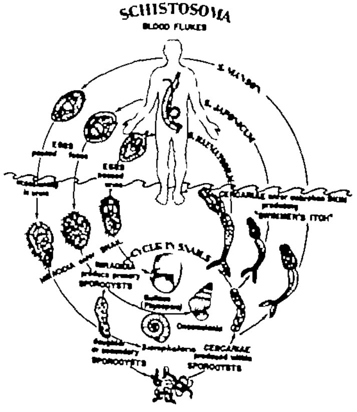

Fig. 2. Diagram illustrating life cycles of three most important species of *Schistosomes* (With acknowledgements to Jordan and Webbe)

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Evidence of Gynecologic Bilharziasis in Cytologic Material

A Morphologic Study for Cytologists in Particular

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One of the scourges of certain countries is bilharzia—a disease caused by a helminth of the genus Schistosoma, which has a life cycle involving two hosts, man and a species-specific aquatic snail.

There are several species of schistosome and the distribution of the three most common species and their life cycle are shown in the following diagrams (Figs. 1 and 2).

It has been recognized for some time that all portions of the female genital tract may be affected by schistosomes.[3, 4, 7, 11, 12, 16, 1, 19]

Gilbert[8] appears to be the first to record the finding of bilharzia ova in "smears" prepared directly from bladder tissue. After the introduction of exfoliative cytology as a diagnostic method, the occurrence of ova in vaginal and cervical smears has been reported by several workers.[1, 17, 21] Reference has also been made in the latest editions of standard books on cytology.[13]

MATERIALS AND METHODS

Since the Cytology Unit of the South African Institute for Medical Research started its work in 1959, bilharzia ova have been encountered from time to time in material examined routinely for detection of neoplasia in the female genital tract. On occasions ova have been present in large numbers, up to 300 in a single smear. Their morphologic appearance has varied considerably depending on their state of preservation, degree of maturation and whether or not therapy has been instituted.

During the years 1962 to 1965 a study was undertaken on selected cytologic and histopathologic material from European, African and Indian females residing in heavily infested areas. This material consisted of 139 cytologic smears containing ova and 374 slides of sections taken from various portions of the female genital tract. The findings were reported previously, and have produced convincing evidence that cytology smears can be of help in the detection of gynecologic lesions, many of which may simulate neoplastic lesions in their clinical presentation, e.g., easily bleeding cervix, ulcerative lesions, nodular wartr growths, fibrosis and induration.

The following five years (1966–1970) have allowed further study of ova in cytologic material. The detailed morphology in Papanicolaou-stained smears is now fully described.

BASIC MORPHOLOGIC FINDINGS

Type of Infection

It has been concluded from the finding of terminal spined ova with certain shape and dimensions in 80 per cent of the material examined that the majority of lesions in the Republic of South Africa are due to *S.* ***haematobium***.

Rare cases do occur of infection with other species of schistosome with terminal spined ova, e.g., *S.* ***mattheei*** which normally causes bovine infection, and *S.* ***intercalatum*** pathogenic to man in the Congo. It is known too, from autopsy studies that *S.* ***mansoni*** does affect the female genital tract.[11, 17] Dwall, working in Natal, has reported finding *S.* ***mansoni*** ova, on one occasion only, in a cervical smear of an African female aged 21 residing in Zululand. Sherman,[17] working in Rhodesia has recently reported finding *S.* ***mansoni*** ova in three smears up to date—one with *S.* ***mansoni*** ova only—two with both *S.* ***mansoni*** and *S.* ***haematobium***.

In this connection, Nelson *et al.*[15] make the point that diagnosis of schistosome species on shape and size of ova is not always reliable since certain factors are known to affect ova: (1) the age of the worms: immature worms sometimes produce very small, distorted ova; (2) the weight of infection: very heavy infestation results in the production of abnormal ova. Gurges and Gelfand[10] report that *S.* ***haematobium*** has a greater egg-laying capacity than *S.* ***mansoni***; (3) the age of the host; and (4) although it is generally assumed that terminal spined ova represent *S.* ***haematobium*** infection, it is of interest to note that seven species of ova have been reported to have been recovered from man in Africa, five of them with terminal spines.[15] It has been mentioned previously that many Africans

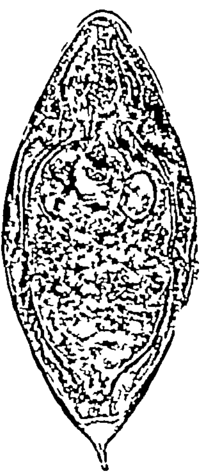

FIG. 5. Photograph of viable unstained ovum of *S. haematobium* with contained miracidium × 800.

relatively undeveloped or well developed when it leaves the uterus of the worm. Within the egg the mature miracidium is capable of very active movements, consequently its anterior end sometimes points in the direction of the spine and at other times away from the spine.

The miracidium of both *S. haematobium* and *S. mansoni* is an elongated boat-shaped organism with a more pointed anterior end and a rounded posterior end. It is an active organism capable of extension and contraction of the body. When extended it can measure 218μ × 29μ and when contracted, 92μ × 55μ. The body is covered with a fairly tough cuticle from which cilia arise. The anterior end has a clearly discernible beak; it is free from cilia and is called the cephalic cone. It is occupied by the digestive tube and the anterior and cephalic glands. Lying just caudal to the digestive tube is the neural centre and a rounded mass of cells containing numerous small dark nuclei which may sometimes be seen in concentric arrangement or even as a ring of peripheral nuclei (lateral gland cells). In the posterior two-thirds of the miracidium the prominent feature is the scattered germinal cells lying loosely arranged in the cytoplasmic background. These germinal cells are even in size and have conspicuous round nuclei, each with a central karyosome giving them a "bull's-eye" appearance.

The miracidia have distinct dorsal and ventral aspects. The neural centre with associated lateral

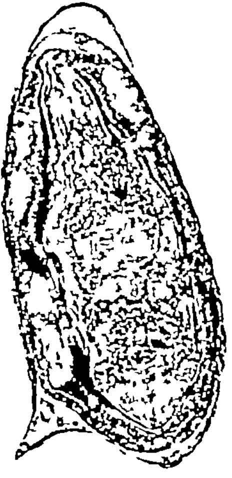

FIG. 6. Photograph of viable unstained ovum of *S. mansoni* with contained miracidium × 800.

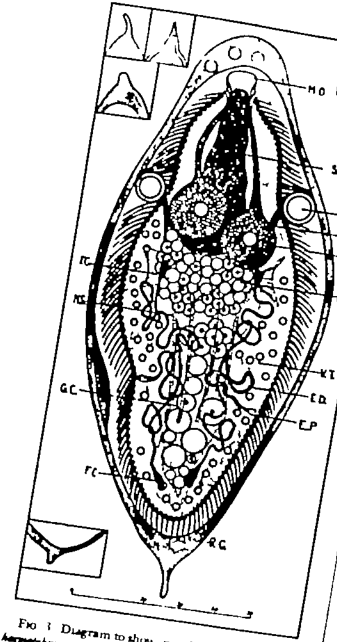

FIG 3 Diagram to show morphology of *Sch tosoma haematobium* ovum ontai ng mat miracid um (with acknowledgements o Girges CG cephalic gland ED excretory duct EP excretory por FC flame cell GC germinal cell LD l teral gland ducts M mucus MO mouth opening NS nervous system NT e runk S gut VT telline tissue Insets arious forms of spine

now resident in the Republic has come from territories outside and may conceivably be infected by schistosomes other than *S haematobium* and *S mansoni* The possibility of hybridization has been reviewed by Pitchford,[29] and Jewsbury reports on host species and geographic trains

Morphology

In order to appreciate the findings in smears, a preliminary account is given of the basic morphology of schistosome ova and miracidia as illustrated in Figures 3 and 4 which are reproductions of drawings by Girges[4] showing ova with their contained miracidia. The descriptions that follow are taken from detailed work done by Girges,[11] and by Porter[14] and Lengy[22]

Schistosome ova when freshly passed are more or less oval structures with a chitinous shell. The ovum of *S haematobium* has a terminal spine, and that of *S mansoni* a lateral spine. The spine may vary considerably in length and sharpness even among ova in the same specimen The ova of both species can vary in length from 80μ to 186μ, and in breadth from 30μ to 93μ In some cases all the ova present are longer than average and in other cases all ova may be predominantly larger or smaller than average Inside the chitinous shell is a thin, colorless vitelline membrane which is usually left behind when the miracidium hatches A clear fluid lies between the shell and the membrane The embryo miracidium inside the egg may be

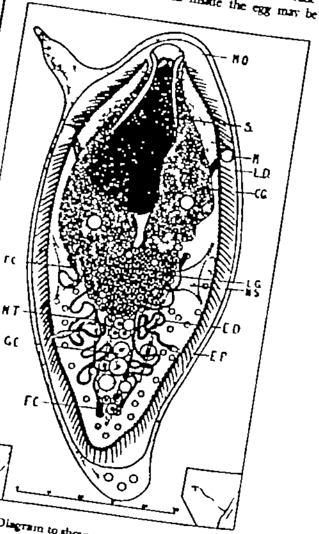

FIG 4 Diagram to show morphology of *Schistosoma mansoni* ovum containing mature miracidium (with acknowledgements to Girges[11]) Labels as in Fig 3.

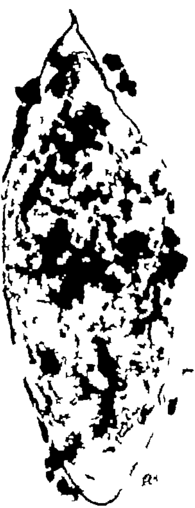

Fig. 9 Mature viable ovum of *S. haematobium*. Cervical smear Pap stain × 600

shell. The outline is symmetrically oval and the spine can be detected. The content appears to occupy the whole ovum and frequently stains basophilically but may stain faintly many color (Figs. 7 and 8).

Immature ova containing a relatively small amount of granular material may be from unfertilized female worms.

(b) *Mature.* A mature miracidium can be seen as a distinct structure within a refractile shell, but when so confined, morphologic detail is difficult to make out. However, the "beak" or cephalic cone, germinal cells and the neural centre can often be seen. As previously mentioned, the cephalic end with the "beak" is sometimes towards the spine, and sometimes away from it. The cytoplasm of the contained miracidia frequently stains deeply eosinophilically but nuclear material of the neural center, lateral gland cells and germ cells stain with hematoxylin, giving definition or producing an over-all granular appearance (Figs. 9 and 10.)

(c) *Hatching or about to hatch.* Prior to hatching, fluid accumulates within the egg which increases in size and the contained miracidium can be seen in attitudes of active contortion. Miracidia escaping from ruptured egg shells can be seen to have expanded in size and morphological detail becomes apparent (see later under Miracidium) (Figs. 11, 12, 13 and 14).

(2) *Degenerate Ova*

The appearance of these ova varies considerably. Some are obviously non-viable and have been dead for a considerable time while others may show

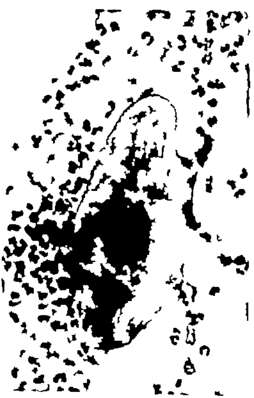

Fig. 10 Mature viable ovum of *S. mansoni*. Cervical smear Pap stain × 600

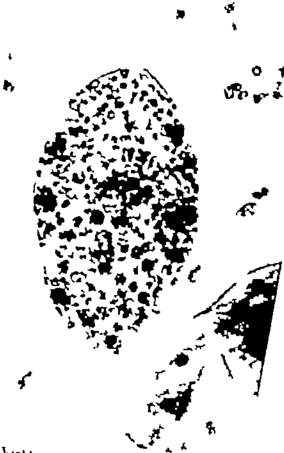

FIG 7 Viable ovum of S. *haematobium* containing immature miracidium with over-all speckled appearance Cervical smear P P stain × 600 (with acknowledgements to J Path Bact 1966)

gland cells, is conspicuous dorsally and th cephalic glands are the more noticeable feature when the miracidium is viewed from the ventral side Small dark nuclei can sometimes be seen just inside the cuticle of the miracidium, these being the nuclei of the outer epithelial cell The germinal cells are seen equally well in both aspects

The shape and activity of miracidia after hatching are reported by Lengy[23] to be dependent on the age and degree of development attained t the time of hatching Porter[8] states that the iracidium of S *mansoni* has a more rugged ap-earance than that of S *haematobium* and the oral ne is more prominent but such differences uld be difficult to make out with certainty in ed stained preparations

Of great morphologic interest are the findings Hockley[14] who reports on the appearance of ova er a scanning electron microscope The whole ce of S *haematobium* and S *mansoni* ova is covered with minute spines These are thought t be associated with enzyme activity and possibl play a part, with the large spine, in transport o ovum from the host to the exterior

Figures 5 and 6 show the shape of fresh, viable, unstained ova of S *haematobium* and S *mansoni*

FINDINGS IN PAPANICOLAOU-STAINED SMEARS

These will be described under the following headings

(1) Viable ova
  (a) Immature
  (b) Mature
  (c) About to hatch
(2) Degenerate ova
(3) Empty egg shells
(4) Free miracidia
(5) Background picture

(1) Viable Ova

(a) *Immature* These have an over-all granular speckled appearance within a refractile chitinous

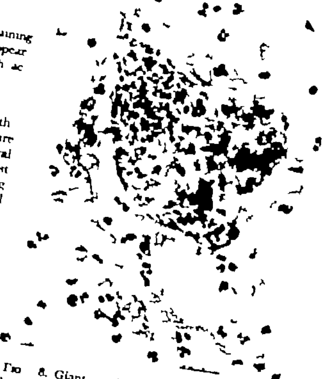

FIG 8. Giant multinucleated histiocytes enveloping ovum of S *haematobium* with immature miracidial contents. Cervical smear P P stain × 600. (With acknowledgements to J P th Bact. 1966)

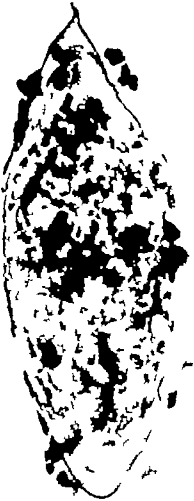

Fig. 9 Mature viable ovum of *S. haematobium*. Cervical smear Pap stain × ,00

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Immature ova containing a relatively small amount of granular material may be from unfertilized female worms.

(b) *Mature*. A mature miracidium can be seen as a distinct structure within a thin refractile shell, but when so confined morphologic detail is difficult to make out. However the "beak" or cephalic cone, germinal cells and the neural centre can often be seen. As previously mentioned, the cephalic end with the "beak" is sometimes towards the spine and sometimes away from it. The cytoplasm of the contained miracidia frequently stains deeply eosinophilically but nuclear material of the neural center, lateral gland cells and germ cells stain with hematoxylin, giving definition or producing an over all granular appearance (Figs 9 and 10.)

( ) *Hatching or about to hatch*. Prior to hatching fluid accumulates within the egg, which increases in size and the contained miracidium can be seen in attitudes of active contortion. Miracidia escaping from ruptured egg shells can be seen to have expanded in size and morphological detail becomes apparent (see later under Miracidium) (Figs 11, 12, 13 and 14).

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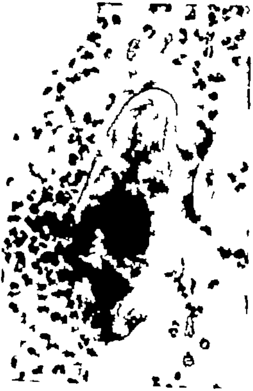

Fig. 10 Mature viable ovum of *S. mansoni*. Cervical smear Pap stain × 600

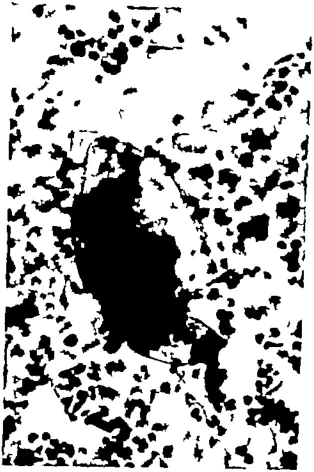

Fig. 11. Ovum of *S. haematobium* about to hatch. Cervical smear. Pap stain × 600 (With acknowledgements to J. Path. Bact. 1966)

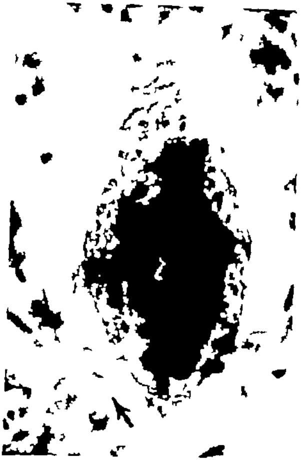

Fig. 13. Miracidium leaving egg shell of *S. haematobium*. Arrow indicates terminal spine. Cervical smear. Pap. stain × 480.

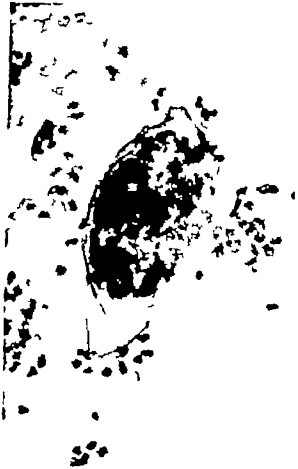

Fig. 1. Ovum of *S. haematobium* about to hatch

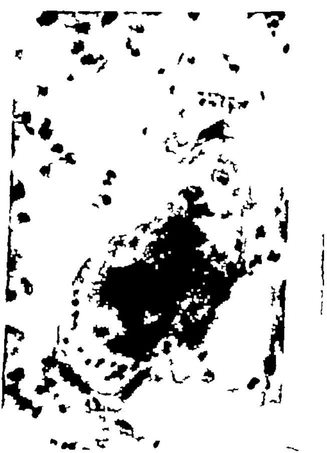

Fig. 14 ing egg she

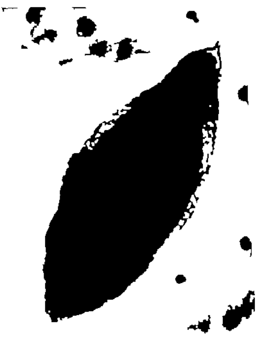

FIG. 15 Degenerate ovum of *S. haematobium* completely black and opaque. Cervical smear. Pap. stain × 600.

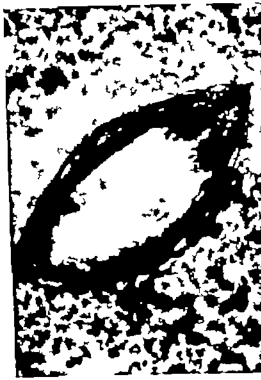

FIG. 16 Degenerate ovum of *S. haematobium*—partially black. Cervical smear. Pap stain × 540. (With acknowledgements to J. Path. Bact. 1966)

some slight morphologic detail suggesting more recent degeneration.

(a) Completely black and opaque. The shape and terminal spine, however, are usually clearly defined. This black appearance, which has previously been attributed to calcification, has recently been reported by Laufer[illegible] to be due to the result of treatment with antimony (Fig. 15).

(b) Black periphery with mauve centre. This distinctly mauve staining reaction with Papanicolaou stain helps considerably with identification of ova (Fig. 16).

( ) Over all mauve with chitinous shell well outlined and contents in which no morphologic detail can be discerned (Fig. 17).

(d) Clearly outlined with shrunken granular contents. The granular appearance in ( ) and (d) is produced by degeneration of the developing miracidium and is described by Laufer[illegible] to occur characteristically after leucanthone treatment (this drug is known to be 100% miracidal). Fixation with alcohol may produce similar effect on immature contents (Fig. 18).

( ) The end result of strenuous treatment is com-

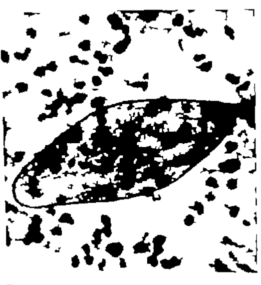

FIG. 17 Degenerate immature ovum of *S. haematobium* in cervical smear. Pap. stain × 600.

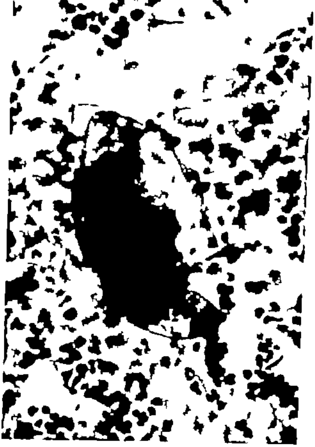

Fig. 11 Ovum of *S. haematobium* about to hatch. Cervical smear Pap. stain × 600. (With acknowledgements to J. Path. Bact. 1966)

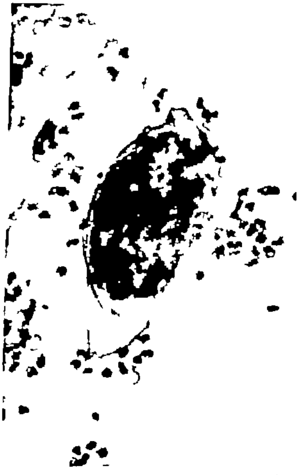

Fig. 12 Ovum of *S. haematobium* about to hatch.

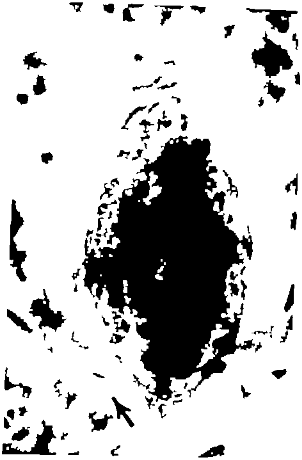

Fig. 13 Miracidium leaving egg shell of *S. haematobium*. Arrow indicates terminal spine. Cervical smear Pap. stain × 480.

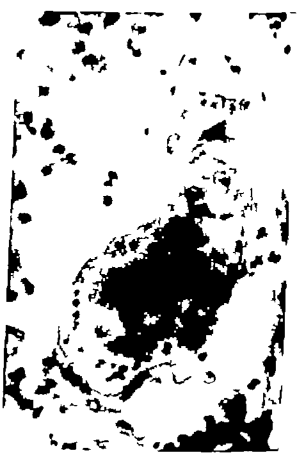

Fig. 14 [illegible] ng egg sh

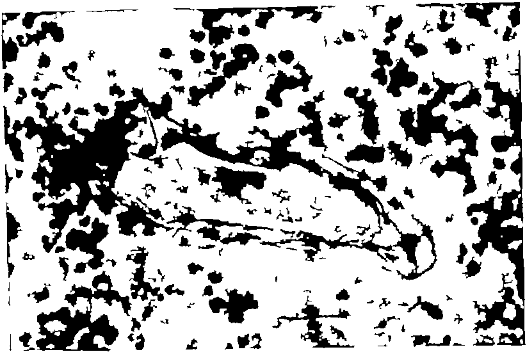

FIG. 20. Crumpled empty egg shell of *S. mansoni.* Note lateral spine. Cervical smear. Pap stain × 600.

pletely amorphous grey ovum. This can only be recognized with certainty in histologic material, but with experience the presence of such structures can be interpreted in cytologic smears.

(3) *Empty Egg Shells*

These have a distinct chitinous refractile outline, but a crumpled look and no structure can be seen within the shell—which has folded edges. Multinucleated histiocytes are frequently seen in the vicinity (Figs. 19 and 20.)

(4) *Miracidia*

These appear in smears as morphologically mature larvae in most instances, but occasionally an immature speckled nondegenerate form is encountered. Their presence is due to rupture of mature ova, either physiologically when in contact with a fluid medium, or as a result of trauma to ova during the process of smear making.

Once free of the confines of the egg shell, the miracidium, which is contractile and an actively motile organism, can assume a number of shapes. It always appears larger than the egg and because it is able to expand, morphologic detail is usually clear. The landmarks described by the early writers are easily recognizable.

Immature miracidia present an over-all speckled appearance in a somewhat basophilic background if well preserved, but appear mauve and granular when degenerate. Mature miracidia have distinctly eosinophilic cytoplasm in which the nuclei of the neural centre, epithelial lining and germinal cell stain conspicuously darkly with hematoxylin.

The neural center may appear as an aggregate of very small darkly staining nuclei or in ring form when the miracidium is viewed from the dorsal aspect. The cephalic glands stain deeply eosinophically but have never appeared in cytologic material to be as conspicuous as they can be in histopathologic sections. Cilia can frequently be seen with careful focusing and are most clearly seen under a phase contrast microscope. The "beak" at the cephalic end is free of cilia. On occasions a "tail" may be seen when the miracidium is contracting. Oval, round and elongated shapes are encountered and can often be well camouflaged by inflammatory cells in the background. The presence of empty egg shells and giant histiocytes indicates the possible presence of free miracidia. A disintegrating miracidium must be distinguished from a giant multinucleated histiocyte. The "bull's-eye" cells of a miracidium are distinctive, as is the intense eosinophilia of the cytoplasm (Figs. 21, 22, 23, 24, 25, 26 and 27).

To be distinguished from a schistosome miracidial larva is another parasite, *Balantidium coli,*

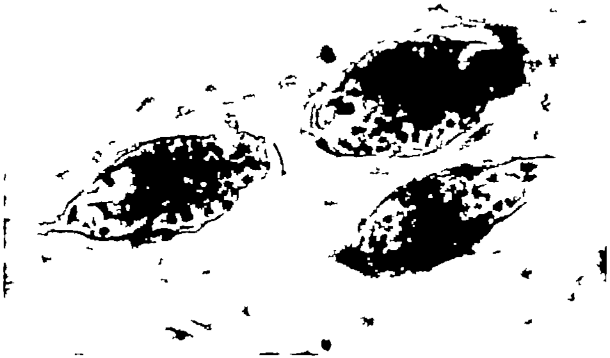

Fig 18 Degenerate immature ova of *S haematobium* with granular shrunken contents. Cervical smear Pap. stain × 540

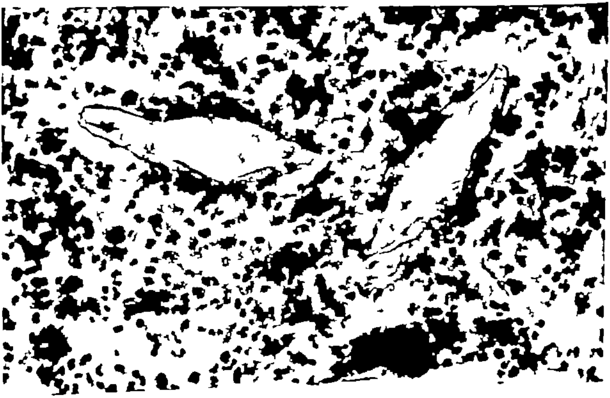

Fig 19 Empty egg shells of *S haematobium* Note crumpled edges and terminal spine. Cervical smear Pap stain × 400 (With acknowledgements to J Path. Bact. 196[illegible])

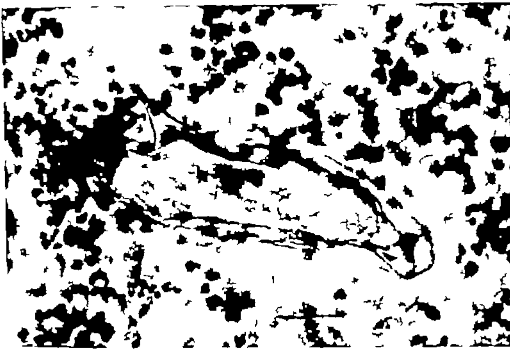

Fig. 20. Crumpled empty egg shell of S. [illegible] Note lateral spine. Cervical smear. Pap. stain × 600

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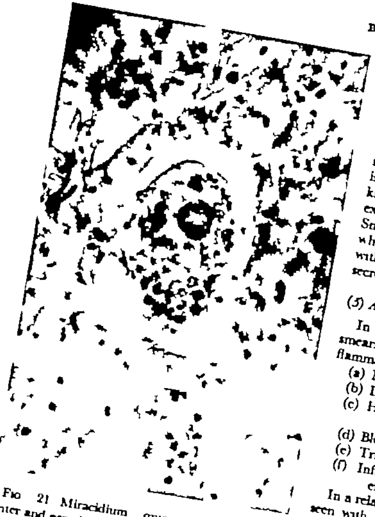

FIG 21 Miracidium quiescent form. Neural center and germinal cells prominent Cervical smear Pap. stain × 480. (With acknowledgements to J Path. Bact. 1966)

which has been reported to cause an infection of the lower female genital tract This is the largest protozoön parasitising man and although normally causing an intractable form of dysentery it is known to be a tissue invader Two records are cited by Faust and Russell of balantidial vaginitis. Mr George Wikeley working in Durban, has recently reported on a case of an African woman of between 40 and 50 years of age residing in Zululand and complaining of a bloodstained vaginal discharge and contact bleeding for several weeks Examination of a vaginal cervical smear made with a gloved finger after pelvic examination revealed in this case literally hundreds of trophozoite forms of *Balantidium coli* and some smaller rounded pre-encysting forms

Morphologic descriptions of this parasite are given by Faust and Russell in both stained and unstained preparations Trophozoite and cyst stages exist. The features described are easily recognizable in Papanicolaou-stained preparations The trophozoite is ovoid in shape, 50μ to 100μ in length and 40μ to 70μ in breadth. The anterior end is somewhat pointed and on one side of the longitudinal axis there is a conical depression, the "gullet" or cytostome. The cytoplasm stains eosinophilically and is finely granular One or more vacuoles may be present. A large, prominent macronucleus staining deeply with haematoxylin is readily identified and may be round, oval or kidney-shaped. A micronucleus which is known to exist is not distinguishable in stained preparations Smaller rounded forms can be recognized in which the parasite has become dehydrated and without completely retracting its cilia starts to secrete a tough cyst wall (Fig 28)

(5) *Associated Cellular Background*

In the material studied the large majority of smears containing schistosome ova showed inflammatory changes which included

(a) Numerous neutrophil leukocytes
(b) Eosinophil leucocytes
(c) Histiocytes including multinucleated histiocytes
(d) Blood
(e) Trichomonads and bacteria
(f) Inflammatory atypia in squamous and endocervical epithelial cells

In a relatively small number of smears ova were seen with concomitant cellular atypia consistent with dysplasia, carcinoma in situ and even invasive squamous carcinoma. These epithelial changes could not, however be attributed directly to the bilharzial infection However considerable epithelial proliferation does definitely occur in association with bilharzial infection and much further work in this direction should be undertaken to establish possible carcinogenicity

The reader is referred to the clinical and pathologic descriptions given by Friedberg[11] and Berry[4] of the related lesions in the female genital tract

Finally the following pictures illustrate for comparison the morphology of other helminth ova which may be encountered in cytological material (Fig 29 30 and 31)

SUMMARY

A full illustrated morphologic description is given of the appearance of bilharzia and other helminth ova in cytologic smears taken from the lower portion of the female genital tract and stained by the Papanicolaou method

ACKNOWLEDGEMENTS

I wish to thank the Director of the South African Institute for Medical Research for permission to publish this paper Professor Crichton and Mr G Wikeley both of Durban Dr Beugger

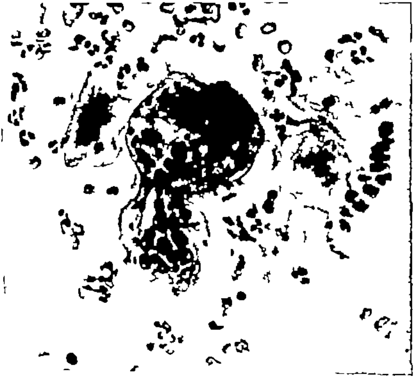

Fig 22 Miracidium motile form Germinal cells and "beak" prominent. Cervical smear P p stain × 480 (With acknowledgements to J Path Bact 1966)

FIG 23. Miracidium prominent beak and "tail" Cervical smear P p stain × 480 (With acknowledgements to J P th Bac 1966)

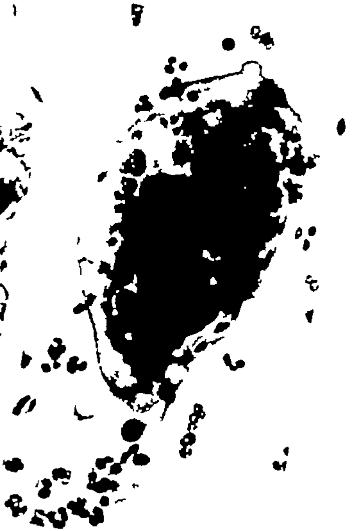

FIG 4 Miracidium. Cervical smear Pap. stain × 480

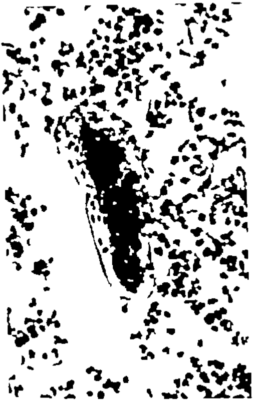

Fig. 25 Miracidium. Cervical smear Pap. stain × 600

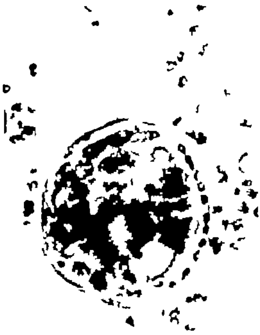

Fig. 26 Miracidium contracted rounded form Cervical smear Pap stain × 480

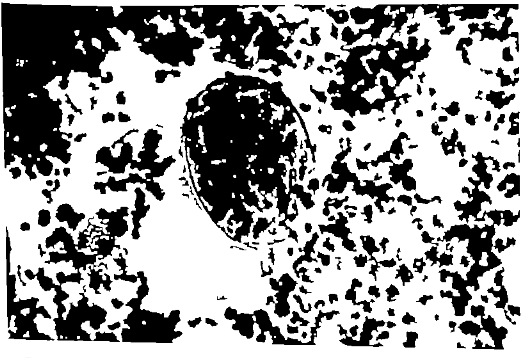

Fig. 27 Miracidium contracted form prominent "tail" and cilia. Cervical smear Pap stain × 480

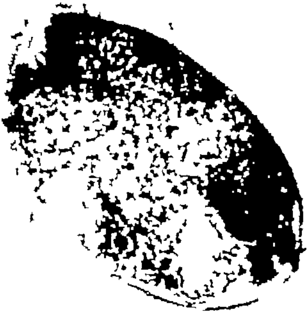

Fig. 28 Balantidium coli ciliated trophozoite form—note kidney-shaped macronucleus and cytoplasmic vacuoles Cervical smear Pap stain × 800

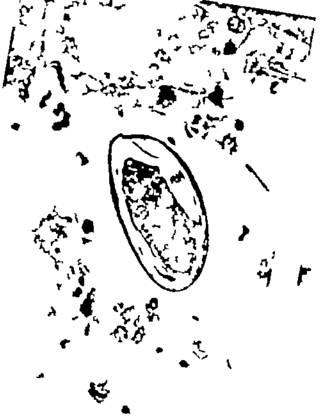

Fig. 29. Ovum of Enterobius vermicularis Note asymmetrical oval shape and absence of spurs Vaginal smear Pap. stain × 600 (With acknowledgements to J. Path. Bact. 1966)

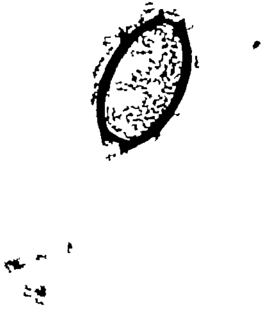

Fig. 30 Ovum

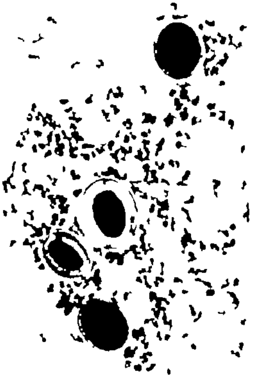

FIG. 31 Terminal ova -outer covering radially striated Cervical smear Pap stain × 480

of Bushbuck Ridge and Mr P Divall of Pietermaritzburg for supplying material from endemic areas M Ulrich in particular for his photography and Mrs R Ross for her assistance

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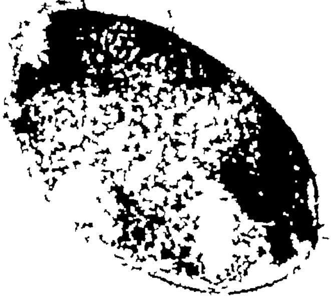

FIG 28 Balantidium coli ciliated trophozoite form—note kidney-shaped macronucleus and cytoplasmic vacuoles. Cervical smear Pap. stain × 800

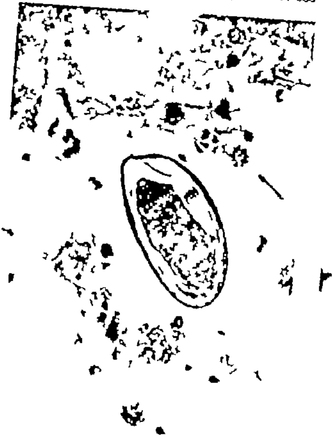

FIG 29 Ovum of Enterobius vermicularis. Note asymmetrical oval shape and absence of spine Vaginal smear Pap. stain × 600 (With acknowledgements to J Path. Bact. 1966).

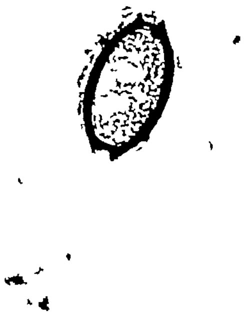

FIG. 30. Ovum of Trichuris trich ur — tea-tray shape. Ce Pap stain × 48

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Cytochemical Determinations of Dry Mass and Protein Content and Cytometry in Bull Spermatozoa

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This the second part of cytochemical study of one population of bull spermatozoa, aimed at determining the *distribution* of major physical and chemical characteristics in this population I the first part[1] we reported on the determination of DNA with the aid of several independent, well studied cytochemical techniques and concluded that measurements representing DNA were significantly more widely distributed in this population of spermatozoa than could be accounted for by methodologic errors

An earlier quantitative study of the masses of bull sperm heads by quantitative electron microscopy had also rendered significant distribution of this property With coefficient of ariation of 15 2% mass distribution was clearly wider than the error of relative dry mass determinations by electron microscopy which is less than ±2

In this second part, series of methods for the cytochemical determination of dry mass and protein content, as well as measurements of size, were applied to bull sperm heads Since the aim the distribution of values rather than their absolute magnitude most of the results are given in arbitrary units only This restriction is particularly relevant in cases in which conversion of relative to absolute values requires either *priori* knowledge of conversion factors or calibration of the technique with model substances N additional information would be gained with respect to the objective of this study by adding bsolute scales

MATERIAL ND METHODS

Preparations of isolated bull spermatozoal heads for this study were made from the same semen sample described in a previous publication and at the time of the first set of preparations

For the measurement of relative amounts of protein, the method of Deutsch was used, which is highly stoichiometric for t-basic groups of lysine, arginine, and histidine Extinction was measured at the second bump of the dye extinction curve at 440 nm (Fig 1) Specimens were mounted in a medium of refractive index 1 556 at 22°C (room temperature) This is the medium at which the unstained spermatozoa of this study could not be seen by either darkfield or phase contrast microscopy

Using Barr and Stroud integrating cytophotometer as described earlier three randomly selected cells were measured 20 times each in order to determine the instrumental error for single measurements This included removal and reinsertion of the object slide and refocusing Thus 300 cells were measured Integrated extinction per cell was determined three times and compared each time with the extinction in its immediate background Averages of three extinction differences (object minus background) were recorded as valid measurement for the cell in question

For quantitative dry mass determinations by electron microscopy suspension of sperm heads was placed on object slides covered with Parlodion (type of collodion) and exposed to 90°C for ten minutes Slides were subsequently washed in 95 per cent methanol in order to remove remaining salts from the suspension on the medium. Sperm heads were photographed at 100 kV using 100 µ objective aperture in Siemens Elmiskop. Exposure and the developing process were controlled in order t produce photographic background density suitable for photometry Integrated transmission of cell images in negatives was determined in an IPM 2 photometer (Carl Zeiss, Oberkochen, Germany)

The relative dry mass of methanol fixed sperm heads, submerged in mixture of equal volumes of glycerin and water was determined in scanning, recording and integrating microinterferometer according to Carlson In this instrument the optical path difference is recorded along

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Constructed for us at the Institute for Cell Research, Karolinska Institute Stockholm (Prof. T Caspersson)

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TABLE III. *Estimation of Error in Dry Mass Determination by Quantitative Electron Microscopy*

	Cell 38	Cell 44	Cell 46
Number of micrographs	20	20	20
Relative mass mean	36.53	45.01	33.71
Variance	0.106	0.491	0.119
Standard deviation	0.326	0.701	0.344
Standard error of mean	0.073	0.157	0.077
Minimum	36.07	42.73	33.24
Maximum	37.03	46.06	34.19
Coefficient of variation	0.9	1.5	1.0

TABLE IV. *Distribution of Dry Mass Determination by Quantitative Electron Microscopy*

	Arithmetic	Logarithmic
Number of cells	328	328
Relative mass mean	43.34	1.6305
Variance	1.140	0.00312
Standard deviation	7.81	0.07152
Standard error of mean	0.432	0.00393
Minimum	31.03	1.4918
Maximum	78.96	1.8952
Coefficient of variation	17.2	4.4
Median	41.3	1.6190

Variance Ratios (arithmetic)

(328)/(20 cell 38)	374.62	
(328)/(20 cell 44)	121.37	P < < 0.001
(328) (20, cell 46)	314.64	in all cases

TABLE V. *Determination of Integrated Optical Path-Difference of Sperm Head By Scanning Interference Microscopy, and Estimation of Error*

	Arithmetic		Logarithmic
Number of measurements	25 (cell)	300 cells	300 cells
Arbitrary integrated O... (mean)	13	5	[illegible]
Variance	96	85	00323
Standard deviation	97	2.42	05680
Standard error of mean	0.19		00328
Minimum	1	1	8737
Maximum		27	378
Coefficient of variation		[illegible]	
Median		2	3004
Variance ratio (300)/(25)	22	P	001

TABLE VI. *Estimation of Error in Determining the Area of Bull Sperm Head from Electron Micrographs by Planimetry*

	Cell A	Cell B	Cell C
Number of measurements	20	20	20
Relative area mean	81.0	88.5	84.3
Variance	1.21	1.52	0.73
Standard deviation	1.10	1.23	0.86
Standard error of mean	0.25	0.28	0.19
Minimum	79.0	87.0	83.0
Maximum	83.0	91.0	86.0
Coefficient of variation	1.4	1.4	1.0

TABLE VII. *Distribution of Area of Bull Sperm Head by Planimetry*

	Arithmetic	Logarithmic
Number of cells measured	500	500
Arbitrary area mean	75.3	1.8738
Variance	28.17	0.00092
Standard deviation	5.31	0.03031
Standard error of mean	0.24	0.00136
Minimum	63.0	1.7993
Maximum	94.0	1.9731
Coefficient of variation	7.0	1.6
Median	75.0	1.8751

Variance Ratios (arithmetic)

(500)/(20 cell A)	23.35	
(500)/(20 cell B)	18.49	P < 0.001
(500)/(20 cell C)	37.70	all cases

Finally we also found measurements of head area having extremely significantly higher variance in the population of 500 than repeat determinations on one cell image. The low coefficient of variation for single determinations agrees well with the low variation in mass determinations and recommends the electron microscope for this type of measurement. Analysis of fit of this distribution to log-normal indicated highly significant lack of fit.

DISCUSSION

Technical

The quality of the sperm sample (one ejaculate) used in this study and in particular the quality of the isolated sperm heads, has been studied in some detail with electron microscopy. Its handling and preparation, as well as the integrating cytophotometer according to Deeley were previously discussed.

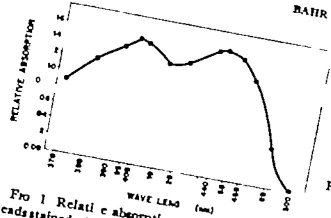

FIG 1 Relative absorption of three bull sperm heads stained with naphtol-yellow-S and measured in a UMSP I (cf ref. ?) at various wavelengths. Curves for individual cells are identical and have not been separated in this graph This absorption curve agrees well with those published by Deitch.[11]

TABLE I *Estimation of Error in Determination of Terminal Basic Group (Protein) By Cytophotometry of Bull Sperm Stained with Naphthol Yellow-S*

	Cell 1	Cell 2	Cell 3
Number of measurements	20	20	20
Relative amount of stain (mean)	4.50	4 76	4 71
Variance	0 031	0 023	0 014
Standard deviation	0 175	0 153	0 118
Standard error of mean	0 039	0 034	0.026
Minimum	4 10	4 60	4 40
Maximum	4 80	5 10	4 80
Coefficient of variation	3 9%	3.2%	5%

TABLE II *Distribution of Cytophotometric Values of Bull Sperm Heads Stained With Naphthol Yellow-S*

	Arithmetic	Logarithmic
Number of measured cells	300	300
Relative amount of stain (mean)	4 66	0 6669
Variance	0 136	0 00122
Standard deviation	0 368	0.0349
Standard error of mean	0 021	0 00202
Minimum	3 53	0 5482
Maximum	5 67	0 7533
Coefficient of Variation	7 9%	5 3%
Median	4 67	0 6690
Variance Ratios (arithmetic)		
(300)/(20 cell 1)	4 46	P < 0.001 in all cases
(300)/(20 cell)	5 80	
(300)/(20 cell 3)	9 74	

equidistant 0 1 μ scanning lines on a strip-chart recorder The area under each scan is automatically integrated. For the purpose of this study only total optical path difference per cell was noted.

Head areas were determined from electron micrographic negatives. Sperm images were enlarged ten times and the outlines traced with a nonpolar planimeter Each cell was measured three times, of which the average was used as input to the statistical analysis.

RESULTS

The numerical results of this study are presented in Tables I to VII In Tables I and II variability of naphthol yellow S stain content among the cells of this population is reported, which proves to be significantly higher than the error of replicate measurements.

Throughout this investigation we found coefficients of variation for repeat measurements in the Barr and Stroud integrating cytophotometer which were < ±6 per cent, in most instances around ±4 per cent.

A comparison of arithmetic mean and median in Tables II indicates no significant difference, suggesting the distribution is not skewed Analysis of skewness, i.e. agreement with a log normal distribution by the method of Cohen for three parameters indicates a highly significant lack of fit.

For mass determinations with the electron microscope, we found an extremely significantly higher variance among 328 cells than for the variance of repeated electron micrographs of a single cell. This is documented by Tables III and IV Coefficients of variation for repeat micrographs are the lowest of any of the determination techniques used in both parts of this study and agree well with earlier detailed discussion of errors in quantitative dry mass determinations by electron microscopy.[4] Differences of mean and median is more pronounced in this series, partly due to the fact that a small number of rather heavy cells give the distribution a tail, up to the maximum value, which is only a little less than twice the mean. There is no second mode and only a faint indication of a shoulder in the distribution around 11×10^{-12} g (Fig 2) Measurements with a scanning integrating interference microscope confirm the trend observed with the two previous methods, variation among 300 sperm cells is significantly higher than among 25 measurements of the same cell.

the diverse production rates of which are very much subject to the ecologic situation of cell, compared with its sister cells. The lower end of the mass distribution would in terms of this line of thinking represent the minimal endowment with proteins required to bring about the supercoiling of chromatin and its compaction into the sperm head. On the heavier side of the distribution one would find cells with significantly more than the minimum amount of nuclear protein, possibly prevented from accumulating more by the normally progressing events of sperm maturation.

Histones are largely unspecifically binding to DNA, there is no protein counterpart to the specificity of nucleic acid base sequences. Only seven histones have been clearly identified for several types are eukaryotes by Bonner group and several non-histone proteins. For bovine spermatozoa, various histone like proteins have been described[7] but we know little about their interaction with DNA, and nothing about whether all or few give rise to the observed quantitative variability. If this variability is due to histone-like proteins, one wonders how the relationship of basic amino side chains to phosphoric acid groups is balanced; more likely also acidic nucleoproteins are involved.

We may presuppose that the *quality* of composition of histone-like proteins is under detailed and strict genetic control through rather well known mechanisms of protein synthesis. Other quantitative data[1] render evidence that genetic control with respect to *quantity* is lax, e.g. macromolecular components of sperm cell are assembled without regard to strict proportionality and acrosoma [illegible] have, quite analogous to this study, significantly wider distribution of mass[4] than is produced by error of the method. Our measurements so far suggest that significant variability of quantity can be expected for biologic entities larger than 5×10^{-18} g or molecular weights over 10.

A major study of intercellular variability has been published by Killander[19] who discusses three causes for distribution of DNA, RNA, and protein among individual cells of population: position of cells in growth and duplication cycle (factor A), momentary variation of cells in the same physiological stage of the cell cycle (factor B), and cell abnormality (factor C). The situation of sperm cells is particular one, generally resembling phase G of the cycle, but with considerable prolongation of this phase in biological stand-by. For normal sperm cells only factor B and differences given by the X and Y chromosomes have to be considered.

Variation in sperm cells has considerably less complex causes, or so it appears, than have been listed for the cells studied by Killander. There is, e.g., no *a priori* reason for assuming that variability of mass is genetically conditioned, because a population of sperm cells would not be genetically mixed in ways other than with respect to distribution of genetic information occurring in meiosis. RNA is very minor component of sperm heads. Variation of RNA and associated proteins could not account for mass distributions with coefficients of variation around 10 and more per cent. Unequal distribution of material to daughter cells or spermatids may be real reason. Its influence on the forming sperm would only be indirect by influencing the cell's post-divisional economy and thus, through feed-back mechanisms, determining rates of synthesis of sperm components.

Variability of amounts of proteins associated with DNA in sperm nuclei (this paper), in chromosomes and metaphases,[27] and at interphase[14] emerges as separate biologic characteristic and is for cells in tissue culture one of the factors producing momentary intercellular variability described by Killander.[19]

Variation of measured parameter has been a common observation in the cytochemical literature, although it has rarely been studied for its own sake. Bredderman and Foote find total spermatozoan volume to range from 15 to 32 μ^3 with an average of 25.2 μ^3 measured in Coulter Counter and in good agreement with earlier published values. These authors observed variability among bulls, especially with respect to susceptibility osmotic changes. In our study, neither naphthol yellow-S measurements nor relative mass by interference and electron microscopy are influenced by the state of swelling of the object; area determinations are, of course, but were obtained from one carefully prepared sample in relative fashion only.

Since spermatozoa have been favorite standards of haploidy in cytochemical work, numerous and diverse measurements have been published[1] from which only few will be reported here because of distributional data contained in them. Sandritter and colleagues measured dry mass for bull spermatozoa with the aid of x-ray microradiography (16 cells, coefficient of variation 5.8%) and interference microscopy (175 cells, coefficient variation 13.1%) and published histogram showing spread comparable to what is reported in this paper. A total mass of 8.88×10^{-12} g has been found with interference microscopy by Sandritter and Grosser. Gledhill in his comprehensive cytochemical studies of bull spermatozoa, collaborated with Carlson and determined the dry mass of 84 cells by x-ray microradiography to 13.2×10^{-12} g, S.D. 1.2 (coefficient variation 9.1%) in good agreement with earlier quantitative electron microscopy measurements. Detailed data on variability of mass and DNA content in bull and

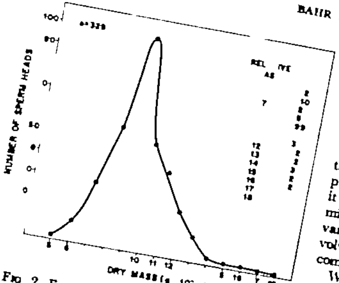

FIG 2. Frequency of occurrence of dry mass of bull sperm heads determined by quantitative electron microscopy The arbitrary scale of mass (abscissa) has been converted to absolute mass with the aid of polystyren mass standards [1] The curve was constructed from remeasured micrographs whereby one more cell was assessed.

It has been reported[illegible] that sperm cells rapidly lose material, mobility and die when suspended in Tyrode's solution. In 62 hours, 38% dry mass had been lost, most in the first eight hours. The sonicated sperm heads of this study appear rather intact in thin sections,[3] and rather comparable to what is seen in freshly fixed cells Sperm heads are not likely to contain large amounts of low molecular material easily lost, neither can they be extracted without severe chemical or mechanical pretreatment Even molar sodium chloride does not extract any nucleoprotein [illegible] We would rather suggest that changes in refractive index of the nucleoprotein have taken place in Tyrode to which the interference microscopic procedure is sensitive and which are not in proportion to changes in concentration In an experiment in which bull spermatozoa of a median weight of 9.62 × 10^{-12} g (97 cells) were sonicated the median weight decreased to 8.62 × 10^{-12} g (101 cells) because the attachment structures of the tail had been removed from the recess in the head's caudal end Storage of this sample for three days at 4°C in a yolk glycine medium did affect neither mass nor distribution of mass in the population a median mass of 8.97 × 10^{-12} g (104 cells) was determined

None of the distributions of measurements could be fitted to a log-normal distribution but neither were they clearly Gaussian, since mean and median were close, but different Some effect from the presence of an X or the smaller Y chromosome in half of the population can be expected Because of the relatively large X in cattle a mass difference can be extrapolated from length measurements suggesting that the average X carrying sperm is likely to be 4.5% heavier than the average Y-carrying sperm.

Biological

Assuming for the sake of the following discussion that the carrier of genetic information in normoploid cells is a constant chemical quantity (cf. 2), it would in the realm of our techniques of determining mass also be a constant mass Any of the variabilities reported in this study could thus involve only the substances and structures accompanying the DNA

With few exceptions, sperm heads form narrow bands in sucrose density gradient centrifugations,[illegible] i.e the cells possess closely comparable buoyant density Variation of mass and of size then means that not only structures enclosing the chromatin can vary but that the head's content must vary along with them, i.e. the proteins associated with DNA must vary

We have no evidence to decide at this point whether the size of a given nuclear membrane—cell membrane—acrosome complex determines or limits the amount of protein entering the sperm head, or if the amount of nucleoprotein is given in each spermatid and the encasing structures adjust accordingly Chromatin fibers seem to arrange themselves largely before nuclear and cell membranes are closely applied to the nuclear content, however which suggests the latter alternative Further direct support for the concept that there is primary variability of nuclear protein is found in measurements of the variability of mass of individual chromosomes and of entire metaphases [illegible] These vary in a way quite similar to findings of this study

No information is so far available to identify the protein or proteins responsible for this variability In Figure 2, weights of sperm heads are presented in a frequency histogram. One could speculate that sperm cells with heads weighing less than 7 × 10^{-12} g or any such cut-off point at the lower end of the distribution, could possibly be defective, although this particular cut-off point would mean that 13.5% of the population were *a priori* produced defective It is still less likely that a cut-off point applies to the upper end of the distribution, because surplus appears biologically more tolerable than the opposite

Before any hard evidence about functional consequences of varying amounts of nuclear protein in sperm cells is available we can surely assume that it is of some consequence and that the most frequently occurring sperm head between 8 and 9 × 10^{-12} g represents the most frequent product of a complex, not necessarily quantitatively well coordinated and controlled, synthetic machinery

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boar spermatozoa have been published by Gledhill and collaborators[16, 17, 18] who come to the conclusion that variation is significant from male animal to male animal and from ejaculate to ejaculate. The notable variability of total mass in the literature is likely to be due to different bulls. Sandritter and colleagues studied 14 bulls, although only a small number of cells are measured in each case the lowest mean is 7 11 × 10^{-12} g[24]

Van Duijn's careful area determinations[26] on bull sperm heads yield a coefficient of variation close to 8.5% as an average for two techniques. Also Paufler and Foote[21] report a range of head areas of bull sperm from 24 9 to 31 5 (our range is 37 6 to 51 5 μ^2 [24])

We must, at this point, for lack of information to the contrary assume that the complex mechanism of fertilization is qualitatively i.e., genetically determined. It will be interesting to see whether future assay methods of a sperm population's fertilizing properties will disclose any significance of the quantitative variability of major sperm components.

SUMMARY

Cytophotometry quantitative electron microscopy and interference microscopy were used to study quantitative variables in a population of cell nuclei with minimum physiologic variation. Spermatozoal nuclei, being in a biologic stand-by phase, were chosen for this purpose.

Dry mass and protein determinations on 328 isolated bull sperm heads reveal for the three independent methods used a statistically high to extremely significant variability as compared to methodologic errors. Comparison of this finding with the literature for bovine spermatozoa indicates that such variability is commonly observed, but has not been studied for its own sake. Momentary intercellular variation of cytochemical values has been the subject of a detailed study by Killander spermatozoan variability is of its own fundamental nature and is only analogous to variability of mass in chromosomes and meta phases, but enters Killander's scheme of causes as an additional parameter The data support the notion that while genetic control in macromolecular assembly of biologic entities with molecular weights larger than 10^7 or weights larger than 5 × 10^{-14} g is qualitatively strict, it is quantitatively lax.

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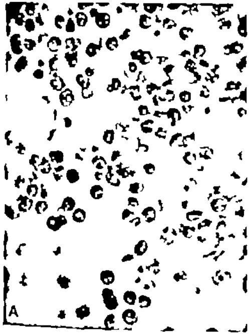

FIG. 2A. Field of leukocytes, histiocytes and degenerating ileal epithelial cells. Papanicolaou Stain, × 449.

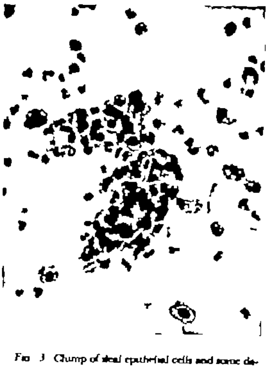

FIG. 3. Clump of ileal epithelial cells and some degenerate ileal epithelial cells. Papanicolaou Stain, × 449.

FIG. 2B. Field of leukocytes, histiocytes and degenerating ileal epithelial cells. Papanicolaou Stain, × 449.

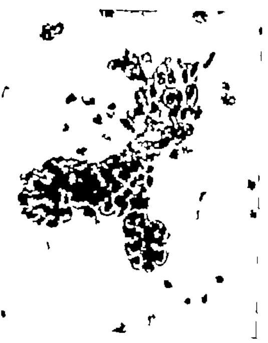

FIG. 4. Clump of ileal epithelial cells. Papanicolaou Stain, × 449.

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Cytology of Ileal Conduit Urine

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The utilization of an ileal conduit has become the preferred method for urinary diversion following resection of the bladder for carcinoma of the genitourinary tract Since these tumors are usually in an invasive stage when cystectomy is employed, close follow up for evidence of recurrent disease is necessary In addition to locally recurrent tumor invading the ileal conduit, a new primary or a previously unrecognized transitional cell carcinoma in the upper urinary tract may occur Cytologic examination of urine from the ileal conduit of these patients has on several occasions provided evidence for the presence of such recurrences Since to our knowledge the cytology of ileal conduit urine has not been described previously and since it differs significantly from normal bladder urine it seemed worthwhile to report Also this simple method offers the possibility of early detection of recurrent cancer with the hope that prompt additional therapy may be of value

MATERIALS AND METHODS

Ileal conduit urine for these studies was collected in the usual external urine reservoir from the ileal conduit stoma and 50 to 100 ml of this urine was submitted immediately to the cytopathology laboratory The urine was passed through a 5μ membrane filter The filter was fixed in modified Carnoy fixative and stained by the Papanicolaou method Occasionally if a great deal of cellular material was present, an aliquot of the specimen was centrifuged and the cell pack fixed in 10 per cent neutral formalin prior to sectioning

RESULTS

The major cellular components of the ileal conduit urine were columnar epithelial cell of the ileum, histiocytes and polymorphonuclear leukocytes Occasional squamous epithelial cells from the skin and transitional cells from the upper urinary tract were also present Tumor cells, when present, revealed the usual characteristics of malignancy including hyperchromatic clumped chromatin, prominent nucleoli, and an increased nuclear cytoplasmic ratio (Fig 1) Bacteria, degenerate cells and debris were also present The amount of cell debris and bacteria varied somewhat from specimen to specimen but was always present in significant amounts and represented one of the interfering factors to examination of these specimens. This feature was in sharp contrast to normal bladder urine and provided a distinctive characteristic of conduit urine The proportion of the various cell types varied somewhat from specimen to specimen, but in general leukocytes histiocytes and degenerate ileal epithelial cells were the predominant cell types (Figs 2A and B) Most often the cells, interpreted as ileal epithelial cells, appeared degenerate with karyorrhectic nuclei and often with fragmented cytoplasm (Fig 3) When these cells were well preserved they were most often in groups and clusters (Fig 4) although they were frequently seen singly as rounded up cells (Fig 5) or as columnar cells (Fig 6) Occasionally slight atypia and vacuolization was evident in these ileal epithelial cells (Fig 7)

A review of the NIH records of urine specimens

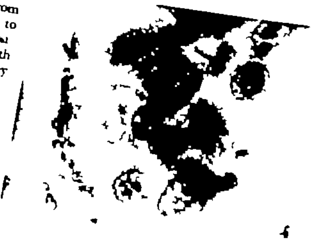

Fig 1 Group of cells from recurrent squamous cell carcinoma of the cervix involving ileal conduit Papanicolaou Stain × 1100

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feature, they do not preclude the detection of neoplastic cells. Debris, presumably from bacteria and degenerate cells, is the main distracting influence which in some instances obscures the cellular details.

These observations and experiences are in contrast to the case described by Sherwood[2] who felt that the ileal conduit urine was not suitable for cytologic study and recommended percutaneous puncture of the renal pelvis to obtain uncontaminated urine. This procedure would, of course, not detect recurrent tumor in the ileal conduit itself.

A hypothetical situation which might make cytologic recognition of recurrent cancer involving the ileal conduit difficult would be a case of adenocarcinoma. The normal ileal cells from the conduit epithelium reveal certain atypical changes suggestive of an adenocarcinoma. These include cytoplasmic vacuolation with nuclear molding and slight hyperchromasia. Since most of the neoplasms involving this region are squamous or transitional cell the problem is not likely to be frequently encountered.

SUMMARY

With the increase of extensive surgery requiring cystectomy for the treatment of cancer involving the genitourinary tract the use of followup cytology for detection of recurrent cancer involving the ileal conduit or upper urinary tract is advocated. A description of the cellular composition of this fluid is presented. Four cases of recurrent cancer involving this area were detected by cytologic examination of the ileal conduit urine.

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Fig. 5 Single rounded ileal epithelial cells and histiocytes. Papanicolaou Stain, × 849

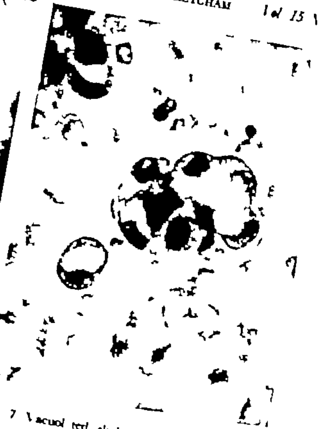

Fig. 7 Vacuolated, slightly atypical ileal epithelial cells. Papanicolaou Stain, × 849

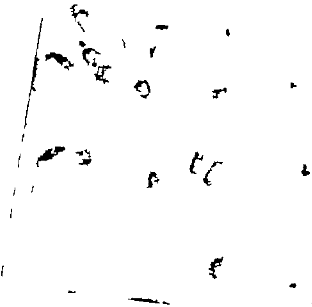

Fig. 6 Single ileal epithelial cells. Papanicolaou Stain, × 849

obtained from ileal conduits between the years 1960–1970 disclosed four cases in which a positive cytology was reported. Two of these patients had recurrent transitional cell carcinoma of the urinary tract while the other two had metastatic squamous cell carcinoma of the uterine cervix. Gross hematuria was the presenting complaint in these cases and the positive cytology was the initial finding confirming a recurrent neoplasm. Exfoliative cytology from the conduit was of added significance when the metastatic carcinoma of the uterine cervix invaded the conduit since the excretory urogram and retrograde ileograms were normal. These radiologic studies were able to identify the site of the recurrent neoplasm in the case of recurrent transitional cell carcinoma. A more detailed description of the clinical course of these patients has been previously described.

Discussion

Examination of ileal conduit urine presents a simple method for follow up study of patients who have had cystectomy for cancer involving the genitourinary tract. This idea was expressed by Koss along with the description of a case seen at his institution. The cytology of this material is relatively straightforward and makes possible early detection of recurrent cancer. Although degenerative changes in the cells are a prominent

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Illustrations. Black and white illustrations will be reproduced free of charge, but the editor reserves the right to establish a reasonable limit upon the number. Colored illustrations will be published at the author's expense. Black and white photographs should be in the form of glossy prints. Line and wash drawings should be black on white paper, with lettering in black india ink, large enough to be readable after necessary reduction. Large or bulky illustrations should be accompanied by smaller glossy reproductions of the same, to facilitate their circulation among the judging members of the editorial board. Photomicrographs should be one column in width (2⅝ inches) so that they may be reproduced without reduction. Illustrations should be numbered, the tops indicated, and the author's name and the title of the article in brief appear on the back. A separate typewritten sheet of legends for the illustrations should be supplied.

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Tissue and Cell Pathology of Uterine Cervix Dysplasias and Carcinoma in Situ

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A variety of intraepithelial alterations have been implicated in the morphogenesis of uterine cervix carcinoma because they were noted either in association with[illegible] or preceding the development of infiltrating carcinoma.[illegible] In retrospective studies, various intraepithelial changes diagnosed prior to invasive carcinoma were interpreted as transitional stages in cervical tumorigenesis.[illegible] In prospective studies of patients with untreated carcinoma in situ[illegible] the occurrence of invasive carcinoma after varying periods of observation ranged from 13.4 to 48.3 per cent.[illegible] The histologic criteria for irreversible precursors to invasive carcinoma have not been defined.[illegible]

The concept of dysplasia as a precursor to carcinoma in situ and invasive carcinoma evolved from observations that various intraepithelial changes preceded carcinoma in situ.[illegible] Other evidence was based upon the frequent occurrence of dysplasia and carcinoma in situ in the transitional zone of the cervix and from the development of carcinoma in situ in areas formerly occupied by subcylindrical anaplasia.[illegible] The presence of histologic fields of dysplasia adjacent to carcinoma in situ[illegible] or surrounding carcinoma in situ[illegible] lend additional support to the possible significance of dysplasia as an early precursor to carcinoma. It was suggested that invasive carcinomas of the small cell type usually arose from subcylindrical cells of the endocervix by way of reserve cell hyperplasia, and that the large cell non-keratinizing carcinoma evolved from the metaplastic epithelium of the transitional zone and the keratinizing carcinoma originated from the squamous epithelium of the portio.

Epidemiologic studies revealed that the incidence rate of carcinoma in situ was much higher in a population previously found to have dysplasia than in a population found to be normal. The average age of patients with dysplasia was about five years less than that of women with carcinoma in situ.[illegible]

In prospective studies of 1922 patients with various forms of dysplasia, 11 of 13 investigators[illegible] reported in 1818 of these cases progression rates to carcinoma in situ from 1.1 per cent to 16.0 per cent and to invasive carcinoma from 0.41 per cent to 1.51 per cent. The lowest rates for regression of dysplasia in this series were 10.8 per cent[illegible] and 20.2 per cent, whereas the remaining cases were associated with regression rates between 32.01 per cent and 53.81 per cent.[illegible] Koss[illegible] reported progression to carcinoma in situ in 42.3 per cent of 26 patients with dysplasia and to invasive carcinoma in 3.8 per cent after an observation period of more than three years. In 38.5 per cent of these patients, dysplasia regressed, whereas persistence was noted in 15.4 per cent. Varga[illegible] found progression from dysplasia to carcinoma in situ in 50.0 per cent and to invasive carcinoma in 14.1 per cent of 78 patients. It was also suggested that invasive carcinoma, particularly the keratinizing carcinoma, may arise directly from dysplasia[illegible] and that microinvasion might originate from basal cells in an epithelium which otherwise appeared normal histologically.[illegible]

The lack of malignant transformation in more than half of the reported cases appeared in keeping with the discrepancies between the number of cases diagnosed as dysplasia and carcinoma in situ and the incidence of invasive uterine cervix carcinoma. In mass cytologic screening projects[illegible] an incidence rate of 82 per 100,000 was determined for dysplasia, whereas carcinoma in situ was found at the rates of 53 and 59.2 per 100,000. In a general population screening project with repeated examination of a total of 32,728 women, cervical biopsies of only 69.0 per cent of 353 patients with abnormal smears led to an annual incidence rate of 218 per 100,000 for carcinoma in situ, whereas annual death rates from invasive carcinoma of the uterine cervix were reported to be 11.5 per 100,000.[illegible]

The discrepancies in the rates of progression from carcinoma in situ to invasive carcinoma and from dysplasia to carcinoma in situ to invasive carcinoma may have occurred as a result of differ

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Volume VI
NERVOUS SYSTEM

Volume VII
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This volume contains 25 papers on defects in the nervous system starting with the clinical approach to disorders and followed by genetic and [illegible] aspects. Also included are 5 case reports.

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Papers include Some R [illegible] Congenital and Metabolic Myopathies, Classification of Neuromuscular Disorders, Hereditary Ptosis. 18 case reports complete the volume.

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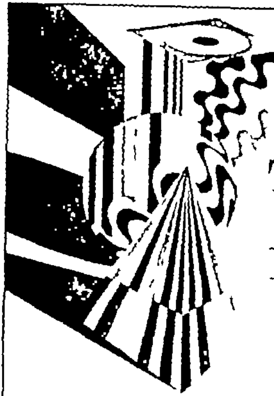

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TABLE II *Comparative Cell Structures in Tissues of the Uterine Cervix and Other Sites*
(Intraepithelial Alterations and Invasive Carcinoma)

Nuclear structure	Benign cell alterations		Malignant cell alterations cervix uteri			Total
	Sites other than ut. cervix	Intraepith. alterations		Squamous cell carcinoma		
		Benign	Malign. neopl.	Invasive	Recurrent	
A	1	1	12	—		16
	0 90%	2 08%	28 57%		15 38%	
AB	20	4	8	3	2	37
	18 02%	8 33%	19 05%	11 54%	15 38%	
B	38	18	—	3	—	59
	34 23%	37 50%		11 54%		
BC	43	19	11	11	5	[illegible]
	38 74%	39 59%	26 19%	42 30%	38 47%	
C	9	6	11	9	4	58
	8 11%	12 50%	26 19%	34 62%	30 77%	
Total	111	48	[illegible]	26	13	240

TABLE III *Cervical Intraepithelial Cell Structure and Polarity Compared with Invasive Carcinoma and Benign Tissues of Other Sites*

Nuclear struct.	Benign cell alterations		Malignant neoplastic cell alterations					Total
	Sites other than ut. cervix	Disorderly Polarity	Orderly polarity		Disorderly polarity			
		Uterine cervix intraepith. alterations				Squam. cell Ca.		
		BD	BD	ICIS	CIS	Invasive	Recurrent	
A	1	—	1	12	—	—	2	16
	0 90%		5 26%	57 15%			15 38%	
AB	20	3	1	7	1	3	2	57
	18 02%	10 34%	5 26%	33 33%	4 76%	11 54	15 38%	
B	38	10	8	—	—	3	—	59
	34 23%	34 46%	42 10%			11 54%		
BC	43	12	7	1	10	11	5	[illegible]
	38 74%	41 39%	36 83%	4 76%	47 62%	42 30%	38 47%	
C	9	4	2	1	10	9	4	58
	8 11%	13 81	10 53%	4 76%	47 62%	34 62%	30 77%	
Total	111	29	19	21	[illegible]	26	13	240

of acute and chronic inflammation, atrophy, hyperplasia, polyps and a variety of benign neoplasms such as papillomas, fibromas, fibroadenomas, leiomyomas and others.

Cell structures in the 111 benign tissues were compared with those in 39 infiltrating cervical carcinomas and with cells in 90 intraepithelial lesions which had loss of differentiation (Table II). The 90 tissues with intraepithelial alterations were then classified according to uniform and disorderly cell polarity and the cellular changes in each of the categories were compared with cells in 39 invasive cervical carcinomas and with those in 111 benign tissues (Table III). Cell structures in the 90 intraepithelial lesions were also studied in correlation with histologic mitoses (Table IV).

RESULTS

Classification of Cell Structures

Study of nuclear structures by utilization of the highest magnification of the light microscope permitted classification of all cellular changes according to three major categories which were designated as cell types A, B, and C. These and their transitional forms AB and BC were encountered in various benign alterations and in malignant tumors. Cells with structureless nuclei, such as the homogeneous pale nuclei and the pyknotic nuclei of necrotic cells, were not included in this classification.

Nuclear structure A consisted of numerous pale round or slightly oval shaped areas, surrounded by curved chromatin bands. Variation ent observation periods. However, the dissimilar rates may have resulted from the inability to determine which of the intraepithelial alterations invariably progress to frank invasion. Koss and co-workers stated that carcinoma in situ of the cervix has a great many faces that are not readily defined or described, and he found no correlation between the behavioral aspect of the lesion and their histologic pattern. Reagan and Wentz were unable to "predict the rate of any given reaction because many of the implicated abnormalities did not ultimate in cancer." They found it therefore impossible to clearly establish the point of no return. Christopherson suggested that carcinoma in situ had diagnostic boundaries which to an extent are debatable and largely subjective.

The inability to define precise criteria for the classification of dysplasia and carcinoma in situ in terms of prognosis led to studies of other parameters for assessment of their invasive potential. Significantly altered DNA values, sex chromatin and chromosomal abnormalities were found in invasive carcinoma and somewhat less consistently in cases of dysplasia and carcinoma in situ. Kirkland's finding of a chromosomal pattern in 27 per cent of in situ carcinomas similar to that of invasive carcinoma, was closely related to the reported progression rates of carcinoma in situ to invasive carcinoma. It seemed that only cases with chromosome patterns similar to those of invasive carcinoma were capable of progression to frank invasion. Therefore the possible reflection of chromosomal abnormalities in mitoses of tissue sections was investigated. The finding permitted in many cases a remarkably accurate estimation of the chromosome number from abnormal mitoses in histologic section of dysplasia, carcinoma in situ and invasive carcinoma.

In turn, the abnormal mitoses and chromosomal patterns could not conceivably occur without prior abnormalities of DNA synthesis and chromosomal duplication during the late interphase possibly with nuclear manifestation in keeping with previously noted nuclear structures in cells of tissue sections and smears. It was therefore anticipated that a comparative study of cells and nuclear structures in benign tissues, invasive carcinoma and intraepithelial alteration may reveal essential parameters for diagnostic classification of cervical lesions.

MATERIALS AND METHOD

All tissue specimens were fixed in formalin. Sections were prepared at 5 micra thickness and stained with hematoxylin and eosin. Previously described cellular changes in tissues with a variety of histopathologic alterations were classified according to nuclei which had either numerous round areas, a prominent nucleolus or chromocenters with band attachments. Alteration in each of these structures were determined by evaluation of nuclear and cytoplasmic size, nuclear cytoplasmic ratio, degree of chromasia, diameters of chromocenters and chromatin band, nucleolar size and nucleolar nuclear ratio (Table I).

TABLE I

DIAGNOSTIC CRITERIA

NUCLEAR STRUCTURES
N, NB, B, BC, C

NUCLEAR CYTOPLASMIC (N/C) RATIOS

Normal or increased. Normal or decreased with marked nuclear and cytoplasmic enlargement. N/C ratios uniformly altered or with marked variation (Anisocytosis and anisokaryosis)

CHROMASIA

Hyper or hypochromasia of nuclear structures N, NB, BC and C

CHROMATIN HETEROGENICITY

Dissimilarity of chromocenters and of number, length and width of chromatin band attachments in nuclear structures BC and C

NUCLEOLAR NUCLEAR (Nu/N) RATIOS
In nuclear structures NB, B and BC

Tissue sections were examined with the light microscope by use of an 3.5X and 10.0X objectives. These magnifications were utilized to determine the type of altered epithelium, degree of epithelial differentiation, arrangement of basal cells, polarity of cells, pleomorphism, and degree of inflammatory cell infiltration. Cellular changes in the tissue were studied with an 100X oil immersion objective. The pathologic significance of cell structures was determined from the histopathologic alteration in the microscopic areas in which the cells were studied.

Benign cellular changes were studied in tissue sections from 111 patients with subsequent long term follow up. The specimens from sites other than the uterine cervix included the respiratory tract, digestive system, genito-urinary tract, skin and breast. The histologic alterations consisted

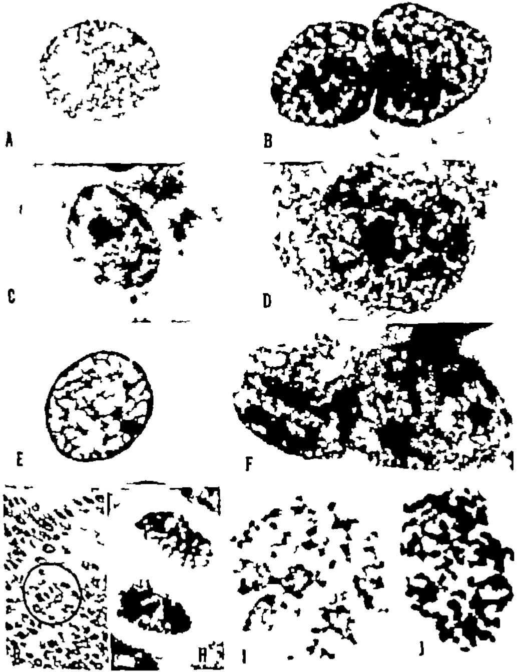


Fig. 1 Benign nuclear structure A. Note numerous round, pale areas surrounded by pale curved chromatin bands (×1250) b Malignant neoplastic nuclear structure A. Note prominent round pale areas surrounded by deeply stained curved chromatin bands (×1 30) Benign nuclear structure B. Note prominent nucleolus, pale chromatin and low N/C ratio (×1250) d Malignant nuclear structure B. Note prominent nucleolus and deeply stained curved chromatin bands. The deeply stained rows of small chromocenters differ from the long chromatin bands of structure C (×1250) Benign nuclear structure C. Note numerous pale chromocenters and bands of varying size (×1250) f Malignant neoplastic nuclear structure C with large, deeply stained chromocenters and bands of varying length (×1230) g Infiltrating carcinoma of uterine cervix (×125) h Higher magnification of Fig. 1g. Note mitotic figure in early telophase with numerous pale round areas surrounded by deeply stained curved chromatin bands (×1230) i Cultured blood lymphocyte in the interphase (×1 30) J Cultured blood lymphocyte in late prophase (×1230)

TABLE IV. *Comparative Cell Structures, Polarity and Mitoses in Benign and Malignant Neoplastic Uterine Cervix Alterations*

Nucl. Struct.	Benign dysplasia				Malignant neoplasia				Total	
	Orderly		Disorderly		Orderly (ICIS)		Disorderly (CIS)			
	No. Cases	Mitoses	No. Cases	Mitoses	No. Cases	Mitoses	No. Cases	Mitoses	No. Cases	Mitoses
A	1	1 (100.00%)	—	—	12	7 (58.33%)	—	—	12	8 (61.54%)
AB	1	1 (100.00%)	3	3 (100.00%)	7	6 (85.71%)	1	1 (100.00%)	12	11 (91.66%)
B	8	—	10	1 (10.00%)	—	—	—	—	18	1 (4.36%)
BC	7		12	6 (50.00%)	1	1 (100.00%)	10	8 (80.00%)	30	15 (50.00%)
C	2	—	4	2 (50.00%)	1	1 (100.00%)	10	8 (80.00%)	17	11 (64.70%)
Total	19	2 (10.51%)	29	12 (41.38%)	21	15 (71.43%)	21	17 (80.95%)	90	46
Total (Orderly and Disorderly)	No. Cases	48				42				
	Mitoses	14 (29.16%)				32 (76.19%)				

in the size of the round or oval shaped areas and of the chromatin bands was inconspicuous or slight (Fig. 1A). This structure was consistently encountered in nuclei of dividing cells during late anaphase and telophase (Fig. 1G, H).

Cells with nuclear structure B had a prominent nucleolus (Fig. 1C) and resembled the dividing cells at interphase (Fig. 1I). The nuclear chromatin in these cells was either not evident or had the structure A or C.

The nuclear structure C consisted of prominent chromocenters which varied in size and had chromatin band attachment of varying number and lengths. The clear spaces between chromatin bands were of diverse shape and size. Often a small number of round pale areas with curved chromatin bands (as seen in A) were also noted in some parts of the nuclei (Fig. 1E). This nuclear alteration closely resembled that of dividing cells in the early prophase (Fig. 1J).

Malignant neoplastic cells of each category had increased nuclear chromasia and increased nuclear/cytoplasmic ratios (N/C) (Figs. 1B, D, F).

Benign Tissue Changes Excluding Uterine Cervix

Examination of benign tissue alterations of various sites from 111 patients revealed cells with nuclear structures A, B and C and their transitional forms AB and BC. Cells with structures A and C usually had low N/C ratios and occasionally increased N/C ratios but consistently had a uniformly pale chromatin appearance (Figs. 2A, B, E, F). In the nuclear structure A the round or oval shaped pale area between curved chromatin bands were not well-defined (Figs. 2A, B). Cells with structure B had a low N/C ratio, delicate appearing chromatin and a low nucleolar/nuclear ratio (Figs. 2C, D). In the combined structures AB and BC the nuclear chromatin was less distinct than in structures A and C and the nucleolar/nuclear ratios were low. These consistently encountered alterations in tissues without tumor led to their classification as benign cellular changes.

Benign cells with structure A were present in 0.90 per cent and those with structure AB in 18.02 per cent of the cases. Cells with nuclear structure B were noted in 34.23 per cent, with BC in 38.74 per cent and those with C in 8.11 per cent of the cases (Table II).

Invasive Uterine Cervix Carcinoma

In tissue sections of 96 cases with invasive carcinoma, the malignant neoplastic nuclear structure A was not encountered in cells of the tumor area. Cells with the combined nuclear structure AB had nuclear hyperchromasia, high N/C ratios, prominent and enlarged nucleoli with or without increased nucleolar/nuclear ratios (Figs. 3C, D). Type B cells had a high N/C ratio, pale chromatin, often markedly enlarged nucleoli with or without increased nucleolar/nuclear ratios (Figs. 3E, F). In the nuclear structure BC the high N/C ratio and the usually enlarged nucleoli was associated with marked hyperchromasia. The type C cells usually had higher N/C ratios than those with nuclear structure B. Evidence of markedly increased hyperchromasia with an increased number of chromocenters and of their chromatin band attachments depended upon thickness and staining of the tissue section. However, variation in size of chromocenters was consistently more conspicuous and chromatin band differed more in number, length and thickness than those in the benign C structure (Figs. 3G, H). Although these changes were often not identified in all cells of the tumor they were always evident in a varying number of

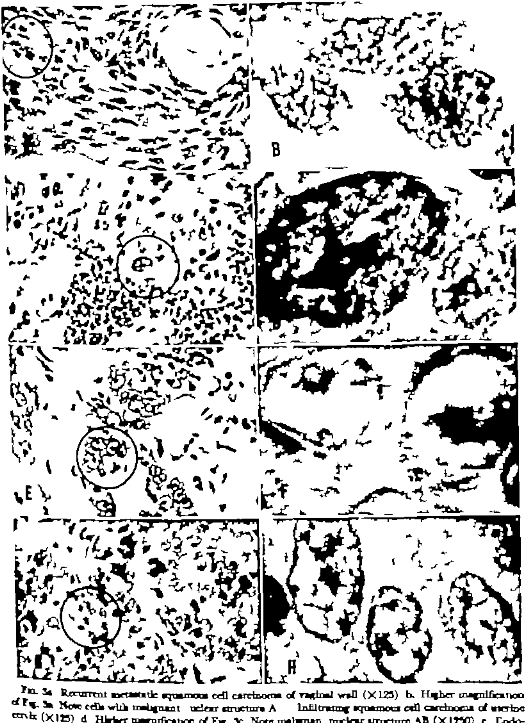


FIG. 3a Recurrent metastatic squamous cell carcinoma of vaginal wall (×125) b. Higher magnification of Fig. 3a. Note cells with malignant nuclear structure A Infiltrating squamous cell carcinoma of uterine cervix (×125) d. Higher magnification of Fig. 3c. Note malignant nuclear structure AB (×1250) e. Endometrial adenocarcinoma (×125) f. Higher magnification of Fig. 3e. Cells with malignant nuclear structure B (×1250) g. Infiltrating squamous cell carcinoma of cervix (×125) h. Higher magnification of Fig. 3g. Cells with malignant nuclear structure C (×1250)

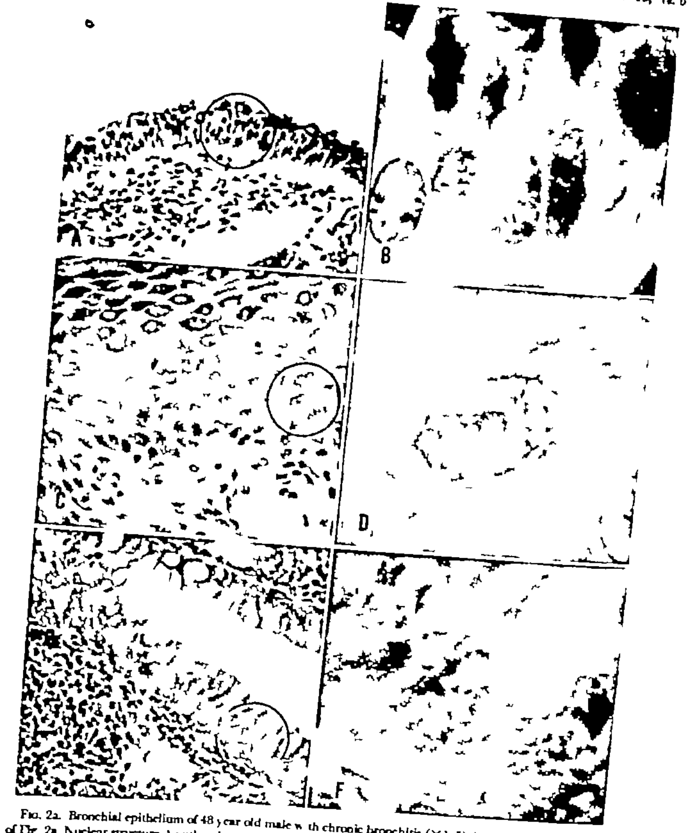


Fig. 2a. Bronchial epithelium of 48 year old male with chronic bronchitis (×125) b Higher magnification of Fig 2a. Nuclear structure A with pal round areas surrounded by pal chromatin bands (×1250) Squamous epithelium of vulva of 57 year old patient (×125) d. Higher magnification of Fig 2c Note cell with benign nuclear structure B (×1250) Area of colonic tissue in case of schistosomiasis (×125) f. Higher magnification of Fig 2e Note columnar cells with benign nuclear structure C (×1250)

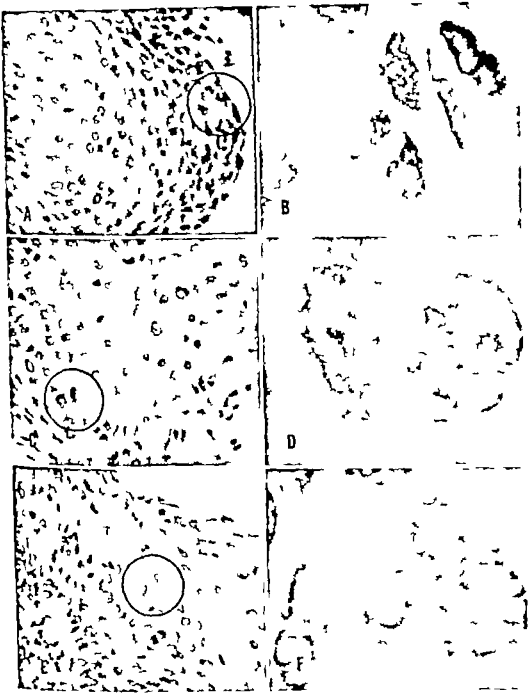


FIG. 4a. Cervical epithelium of cone from 38 year old patient with benign dysplasia (×125). b. Higher magnification of Fig. 4a. Cells with benign nuclear structure A (×1250). c. Cervical epithelium of 25 year old patient with benign dysplasia and evidence of chronic cervicitis in adjacent areas (×125). d. Higher magnification of Fig. 4c. Cells with benign nuclear structure AB (×1250). e. Cervical epithelium of 2[illegible] year old patient with benign dysplasia (×125). f. Higher magnification of Fig. 4e. Cells with benign structure C (×1250).

cells, and consistently absent in benign tissues. They were therefore classified as malignant neoplastic alterations.

Cells with the combined nuclear structure AB and with structure B were noted each in 11.54 per cent of the cases Cells with the combined structure BC were found in 42.30 per cent, whereas tumors with type C cells constituted 31.62 per cent of the invasive carcinomas (Table II)

In tissue sections with recurrent carcinoma, the cells with nuclear structure A were often noted in areas with uniform cell polarity The nuclear structure consisted of more clearly defined pale round areas with usually greater uniformity of shape and diameter than in the benign tissues The curved chromatin bands around the pale area were usually more deeply stained than in benign cells The N C ratio was markedly increased often as a result of decreased cytoplasmic size rather than as a result of nuclear enlargement (Figs 3A, B) The moderately enlarged nuclei were usually smaller than the occasionally noted large nuclei with structure A of benign cells.

In 13 recurrent carcinomas of the uterine cervix, 15.38 per cent of the cases had cells with the malignant nuclear structure A. Cells with structures AB were present in 15.38 per cent, whereas the nuclear structure B was not encountered in this group of recurrent early carcinomas. In the remaining cases 38.47 per cent of the tumors had cells with structures BC and 30.77 per cent had cells with structure C (Table II)

Cell Structure in Cervical Intraepithelial Alterations

In 48 of 90 tissue sections with intraepithelial loss of differentiation, the nuclear structures were comparable to those of benign tissues. The benign nuclear structure A (Fig 4A, B) was present in 2.08 per cent of the tissues, AB in 8.33 per cent (Fig 4C, D) B in 37.50 per cent BC in 39.59 per cent and C (Fig 4E, F) in 12.50 per cent of the cases (Table II) Resemblance of cell structures to those of benign tissues from other sites led to the classification of these intraepithelial alterations as Benign Dysplasias

In the remaining 42 tissue sections the cells had malignant nuclear structures and increased N C ratios (Figs 5A D and 6A D) Nuclear structure A was noted in 28.57 per cent and AB in 19.05 per cent Cells with structure B were not encountered in these cases. Malignant structures BC and C were present each in 26.19 per cent of the cases (Table II) These cellular changes were identical with those of invasive carcinoma (Figs. E, F and (T F) Therefore the epithelial alterations were interpreted as "intraepithelial malignant neoplasia"

Nuclear Structures in Relation to Cell Polarity

Benign Dysplasia In 48 tissues with benign dysplasia cell polarity was disorderly in 29 cases and orderly in 19 (Table III) In 29 tissues with disorderly polarity cell type A was not present. Nuclear structures AB were noted in 10.34 per cent, structure B in 34.46 per cent, BC in 41.39 per cent and C in 13.81 per cent of the cases. In tissues with orderly cell polarity the nuclear structures A and AB were each found in 5.26 per cent, structure B in 42.10 per cent, BC in 36.85 per cent, whereas cell type C was noted in 10.53 per cent of the specimens.

Carcinoma in Situ. Cell polarity was disorderly in 21 of 42 tissue sections with malignant cytologic changes. The malignant nuclear structures A and B were not encountered in these cases and the combined structure AB was found in only 4.76 per cent of the 21 specimens. Cells with malignant structures BC and C were each present in 47.62 per cent of the cases (Table III) These intraepithelial alterations with loss of differentiation, disorderly polarity and malignant neoplastic cells were interpreted as "Carcinoma in Situ" because their histologic architecture and cell structure (Figs. 5A–D) were identical with those of infiltrating carcinoma except for stromal invasion (Figs 5E, F)

Incipient Carcinoma in Situ. In the remaining 21 cases, the malignant neoplastic cells had an orderly polarity Cells with type A nuclei were present in 57.15 per cent (Figs. 6A, B) and those with type AB in 33.33 per cent (Figs. 6C, D) of the tissues. Cells with structure B were not found and structures BC and C were present each in 4.76 per cent of the cases. In this group of cases, cells with malignant nuclear structure A were comparable to those of some infiltrating carcinomas in other sites (Figs. 6E, F) and to cells in areas with orderly cell polarity of recurrent cervical carcinomas (Figs. 3A, B) The other structures were also identical with cell alterations of invasive carcinomas However 90.48 per cent of the intraepithelial lesions with uniform polarity had malignant cells A and AB in marked contrast to the epithelia with disorderly cell polarity of carcinoma in situ which in 95.24 per cent of the cases consisted of malignant cells BC and C (Table III)

The malignant neoplastic intraepithelial lesions with predominantly A and AB nuclear structures and uniform cell polarity were designated as "incipient carcinoma in situ" (ICIS) and distinguished from benign dysplasia by their malignant cell alterations and from carcinoma in situ by their nuclear structures and polarity

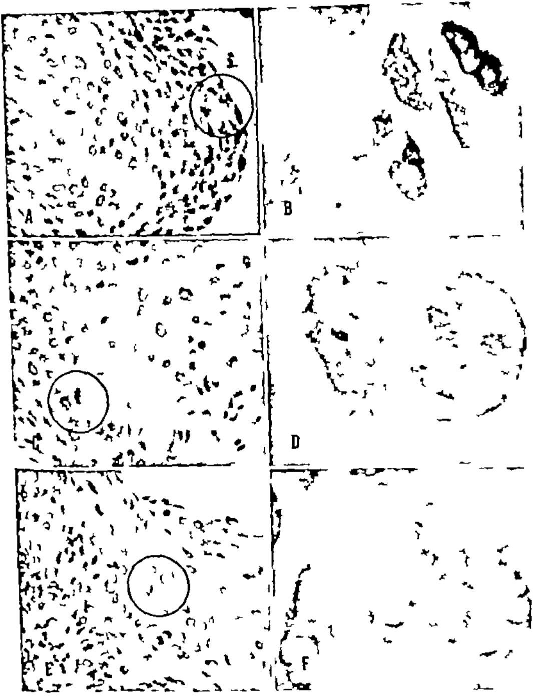


FIG. 4. Cervical epithelium of cone from 38 year old patient with benign dysplasia (×125). b. Higher magnification of Fig. 4. Cells with benign nuclear structure A (×1250). Cervical epithelium of 23 year old patient with benign dysplasia and evidence of chronic cervicitis in adjacent areas (×125). d. Higher magnification of Fig. 4c. Cells with benign nuclear structure AB (×1250). Cervical epithelium of 29 year old patient with benign dysplasia (×125). f. Higher magnification of Fig. 4e. Cells with benign structure C (×1250).

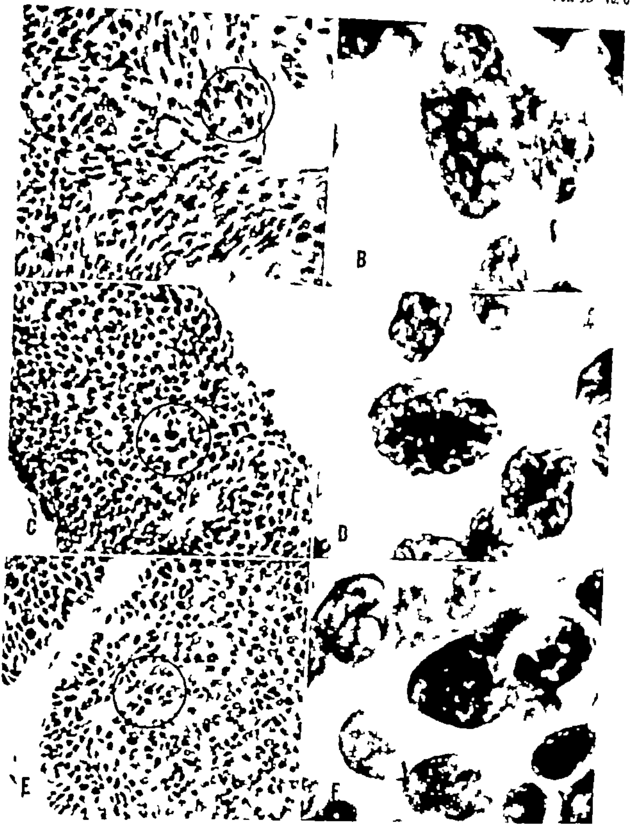


Fig. 3a. Cervical cone of 47 year old patient with carcinoma in situ. Note area with disorderly cell polarity (×125). b. Higher magnification of Fig. 3a. Cells with malignant nuclear structure C (×1250). c. Carcinoma in situ in cervical biopsy 2 years prior to diagnosis of invasive carcinoma (×125). d. Higher magnification of Fig. 5c. Cells with malignant nuclear structure C (×1250). e. Infiltrating squamous cell carcinoma of uterine cervix (×125). f. Higher magnification of Fig. 3e. Cells with malignant nuclear structure C (×1250).

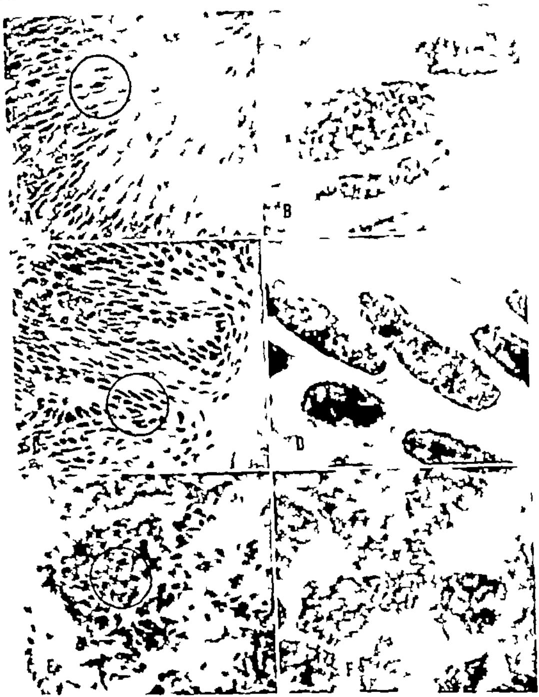


Fig. 6a. Incipient carcinoma in situ in cervical cone from 38 year old patient. Note loss of differentiation and uniform cell polarity (×125). b. Higher magnification of Fig. 6a. Cells with malignant nuclear structure A (×1250 Incipient carcinoma in situ in cervical cone from 48 year old patient. Note uniform cell polarity and surface differentiation (×125). d. Higher magnification of Fig. 6c. Cells with malignant nuclear structure AB (×1250). e. Fragments of bronchial mucosa with infiltrating small cell carcinoma from 51 year old female (×125). f. Higher magnification of Fig. 6e. The tumor cells have malignant nuclear structure A (×1250).

Cell Structures and Polarity in Relation to Histologic Mitoses

The presence and phase of mitoses was examined in 90 cases with benign and malignant neoplastic intraepithelial changes (Table IV) In 19 benign dysplasias with orderly cell polarity 10.53 per cent of the cases had cells in metaphase which were exclusively encountered in tissues with nuclear structures A and AB In 29 benign epithelial dysplasias with disorderly cell polarity cells in metaphase were present in 41.38 per cent of the cases. Mitoses were frequently noted in tissues with nuclear structures AB (100.0%) BC (50.0%) and C (50.0%) and were rarely found in those with structure B (10.0%)

In 21 intraepithelial malignant neoplasias with orderly cell polarity (incipient carcinoma in situ) anaphase and telophase forms were noted in 71.43 per cent of the cases

In intraepithelial malignant neoplasias with disorderly cell polarity (carcinoma in situ) mitoses were usually asynchronous in anaphase telophase and metaphase and were present in 80.95 per cent of the cases (Table IV)

Histologic Intraepithelial Patterns Nuclear Structures and Alterations in Relation to Biologic Behavior

Initial intraepithelial alterations from cases with long term follow up information were reclassified on the basis of cell structures and polarity into benign dysplasia, incipient carcinoma in situ and carcinoma in situ (Table V)

In 18 cases with benign dysplasia, the majority of tissues consisted of cells with nuclear structures B and BC. The cell polarity was orderly in 7 and disorderly in 11 cases. Mitoses in the *prometaphase and metaphase* were found in one case with orderly polarity and in four dysplasias with disorderly polarity Benign dysplasia had persisted in 16 of these patients after an average observation period of seven years and nine months The remaining two cases with disorderly polarity developed incipient carcinoma in situ after an average observation period of seven years and six months.

Incipient carcinoma in situ was present in seven initial tissue sections which had uniform cell polarity The cell structures were type A in three AB in three and type C in one case Mitotic figures in anaphase and telophase were noted in three cases with type A and AB cell structures and in the case with type C cells. After an average observation period of four years and three months, carcinoma in situ occurred in all seven cases. Following continued observation of six of these patients, persistence of carcinoma in situ was noted in two cases after a period of five years whereas four patients had invasive carcinoma after an additional average observation period of four years.

In eight cases the diagnosis of carcinoma in situ was based upon presence of malignant neoplastic cell structures and disorderly cell polarity Cell structure BC was noted in 4 cases and structure C in the other four Mitoses were present in

TABLE V *Comparison of Cell Structures and Polarity in Morphogenesis of Uterine Cervix C*

Initial findings							Subsequent diagnoses				Total		
		Benign cell structures											
Intraep alter	Polarity	A	AB	B	BC	C	BD	ICIS	CIS	Inv C	Others	period	No cases
BD	Orderly			3			5					7y 5m	5
					2							6y	
			1	1								7y 6m	
	Disorderly			7			7					7y 9m	7
												9y 6m	
Total			1	13	4		16						18
		Malignant cell structures											
ICIS	Orderly	3								1		6y 4m	3
												4y 6m	
			1							1		9y	1
						1			1			9y	1
Total		3	3			1			3	4			7
CIS	Disorderly				4					4		3y 9m	4
						4				4		3y 6m	4
Total					4	4				8			8
Total		3	4	13	8	5	16		3	8			33

75 per cent of the cases. In four patients, invasive carcinoma of the uterine cervix was found after an average observation period of three years and nine months. In the remaining four patients, invasive carcinoma was evident after an average observation period of three years and six months.

DISCUSSION

Nuclear Structures

Diagnosis of histopathological changes by additional utilization of high microscopic magnifications for evaluation of cell structures and their alterations differs from classifications which are based upon criteria for pattern recognition at low magnifications. Alterations in the nuclear chromatin structure do not occur in an arbitrary manner as result of fixation, but rather reflect the state of chromosomal development at the time of fixation.[17] Similarity of nuclear structures A, B and C with telophase, interphase and prophase of dividing cells in culture and the close relationship between changes of these structures and that of the histologic pattern may represent manifestations of the cytogenetic factors in the morphogenesis of disordered tissue growth.

In cell type A the pale circular areas in the nuclei were described by others as nuclear vesicles in the late anaphase and telophase.[illegible] This cell type with malignant neoplastic alterations corresponds apparently to the nuclear structure reported by Atkin[3] as condensed telophase chromatin patterns in cells of human tumors. Interpretation of cell nuclei as telophase structures was made from comparative evaluation of DNA values with cells in the interphase of the same tumor. The same telophase structure observed in larger nuclei was apparently related to increased DNA values in keeping with the hyperdiploid and hypotetraploid chromosome patterns of the tumor cells. In cell type B the prominent nucleolus and the delicate nuclear chromatin of the normal cell is comparable to interphase nuclei in cultured cells. In cells with nuclear structure C the small chromocenters were interpreted as telomere attachments to the nuclear membrane during the prophase, whereas chromatin bands apparently represent the chromatin threads which condense into chromosomes at later stage of the prophase. Observations by time lapse studies of these chromatin threads in the prophase of living mouse nuclei were reported as an indication of the close relationship between nuclear structures and chromosomal development. This relationship between mitotic phases and nuclear alterations indicates that the combined structure AB reflects transition from telophase to interphase (G1) and the structure BC occurs probably between G1 and S phase, whereas structure C is apparently related to the S and possibly also G2 phase.

Comparative evaluation of this classification with existing terminology is difficult because previous descriptions of cell alterations were based upon observation at low magnifications. Thus, "nuclear stippling and uniformly or finely granular" nuclei, observed at low power may appear at high magnification as the uniformly small chromocenters which surround the pale circular areas in the nuclear structure A. Similarly clumping of the chromatin and "parachromatin clearing" may be evident in the nuclear structure C as chromocenters of varying size and clear spaces between chromatin bands, whereas the coarsely granular" chromatin of malignant cells may correspond to either structure A or C.

Relationship of Cell Structures to Histologic Pattern

The observation that cell alterations are closely related to changes of the tissue architecture is in keeping with the reported relationship of DNA values[1] [illegible] and chromosomal abnormalities[illegible] [illegible] [illegible] to histologic changes. Although malignant tumors usually contained varying number of benign appearing cells, the consistent presence of numerous malignant neoplastic cell structures and their absence in benign tissues was interpreted as evidence that malignant neoplasia does not occur without corresponding cell alterations. On the assumption that malignant neoplastic cell structures reflect abnormal chromosome patterns and DNA values, it is difficult to conceive that these changes take place as result of histologic alterations. It seems more likely that malignant cell transformation precedes the histologic changes during the course of malignant tumor development.

In addition to different alterations of the same nuclear structure, depending upon their benign or malignant role, the predominant presence of particular cell types in relation to different histopathologic changes is of interest (Tables II and III). The largest number of benign tissues consisted of cell types B and BC, whereas the majority of cases with carcinoma in situ and invasive carcinoma had malignant cells BC and C, whereas 90 48 per cent of incipient carcinoma in situ had nuclear structures A and AB. This consistent relationship of altered cell structures to histologic changes seemed to offer fundamental parameter for assessment of potentially malignant neoplastic intraepithelial cervical alterations.

Thus, in cervical tissues with loss of intraepithelial differentiation and either uniform or disorderly polarity, lack of malignant nuclear changes and presence of cells comparable to those of benign tissues from other sites, led to the diagnosis of benign dysplasia. Intraepithelial malignant neoplasias with uniform cell polarity (incipient carcinoma in situ) were distinguished from carcinoma in situ with disorderly polarity in which the cells with predominantly BC and C nuclear structures had the same alterations as those of invasive carcinoma As a result of the close resemblance of cells in carcinoma in situ to those of invasive carcinoma, differentiation between invasive and noninvasive carcinoma has not been possible in smears on the basis of cell types alone (50 74) The malignant nuclear structure A in cells of incipient carcinoma in situ, though absent in carcinoma in situ and invasive carcinoma, was present in some infiltrating tumors of other sites and in areas with uniform polarity of recurrent cervical carcinomas. Presence of cells with malignant nuclear structure A therefore indicates the malignant potential of incipient carcinoma in situ and probably also reflects a transitional stage in the morphogenesis of recurrent carcinoma

There is no satisfactory explanation at present for the consistent association of particular cell structures with the type of histologic alteration. Based upon the relationship of chromatin patterns to the phase of mitoses, the various nuclear structures may occur as a result of delays in different mitotic phases. In the incipient carcinomas in situ therefore, the predominant presence of cells with nuclear structure A may reflect a stage of tumorigenesis in which the oncogenic action upon cells produces a mitotic delay of the telophase whereas in carcinoma in situ and invasive carcinoma, the cell prior to division are subject to delayed S or G2 phases. Any relationship of this phenomenon to either duration of malignant transformation or to chromosome patterns and DNA values remains to be determined

Nuclear Alterations and Cell Polarity in Relation to Histologic Mitoses

Mitotic figures may be found in normal and regenerating epithelia as well as in inflammatory processes. Occasionally mitoses may be abnormal in these benign conditions. Tissues of malignant tumors, however are frequently associated with abnormal mitoses. The larger number of cases with mitoses (76.19%) encountered with intraepithelial malignant neoplasias than with benign dysplasias (29 16%) supports the validity of the cytologic criteria for the diagnosis of these lesions. Although incipient carcinoma in situ differed from carcinoma in situ by nuclear structure and cell polarity both had a similar proportion of cases with mitoses (71 45% in incipient carcinoma in situ and 80.95% in carcinoma in situ) Other distinguishing features consisted of anaphase and telophase mitotic figures in incipient carcinoma in situ and of apparently asynchronous mitoses with metaphases in carcinoma in situ. However in both the chromatin structures of the dividing cells were similar to those of invasive carcinoma. It therefore seems that incipient carcinoma in situ and carcinoma in situ are potentially malignant neoplastic alterations and that changes in their cell structures, polarity and type of mitoses are apparently related to different stages of malignant transformation.

Contrary to anticipated results, mitotic figures were found more frequently in benign and malignant intraepithelial alterations, which consisted of cell type AB (91 66%) than in those which had other nuclear structures.

Unlike intraepithelial malignant neoplasias, the benign dysplasias with uniform and disorderly polarity had the same nuclear structures but differed by their incidence of mitoses. In benign dysplasias, mitotic figures were more often observed in tissues with disorderly polarity (41.38%) than in those with uniform cell polarity (10 53%) These findings may have several implications. The well defined alterations of incipient carcinoma in situ and carcinoma in situ were identified as arbitrary landmarks at separate stages of tumor development and are therefore readily distinguished from each other and from benign dysplasia. While transitional changes are usually prominent during malignant transformation at a relatively advanced stage between incipient carcinoma in situ and carcinoma in situ, the transition between benign dysplasia and incipient carcinoma in situ is often subtle On the assumption that the first discernable epithelial manifestation of malignant neoplasia does not arise de novo from normal epithelium, it is conceivable that some benign dysplasias may have a potential for malignant transformation Correlation of various parameters in this study revealed several differences in their alterations. Thus benign dysplasias may be distinguished according to those with mitoses and cells A or AB in uniform polarity those with mitoses and cells BC or C in disorderly polarity and dysplasias with cells B, BC or C in either uniform or disorderly polarity without mitoses (Table IV) Possibly this subclassification of benign dysplasias may lead to identification of the alterations which precede incipient carcinoma in situ.

Incidence and Progression Rates of Dysplasias and Carcinoma in Situ

Intraepithelial lesions found by periodic reexamination of patients previously known to be

TABLE VI *Prospective Studies of Dysplasia*

	Observation Period	Regression	Persistence	Progression		Comments	Total
				CIS	Inv. Ca.		
[illegible] (18)	4y	37 08%	52 81%	10 90%	—		148
Johnson et al (21)	Under 6m to 6y and over	40 44%	58.28%	12%	—		89
Johnson et al (2)	Up to 9y	50 40%	48 20%	1 48%	—		141
Jordan et al (27)	2m–11	44 29%	20 08%	6.42%	—	23 of 95 pts. were lost to follow up	95
Kim et al (34)	3y	38 50%	13 40%	42.30%	3 00%		78
Lambert (37)	2m–3y	46 29%	25 14%	12 08%	—		108
McKay (38)	10y	20 20%	38 40%	0%	0 77%	(Of 8% converted with CIS or Inv Ca)	129
Peckham et al (57)	Under 12m to 48m & over	40 53%	48 80%	1 70%	—		348
Reagan et al (37)	1½ to 4y	53 80%	30 70%	13 80%	1.30%		83
Richard (60)	2m–2 5y	10 80%	—	90%	—		80
Richard (61)	3y	—	28 30%	16 00%	—	(% progression per yr)	557
Stern and Neely (70)	6m–9y	22 08%	—	40%	6 40%		190
Varga (73)	6y	—	35 80%	50 00%	14 10%		78
							1 922

free of disease do not necessarily reflect incidence rates. In repeated screening of population groups, the interpretation that newly found cases of dysplasia and carcinoma in situ had developed subsequent to the last examination depends upon several factors. The time interval between the two examinations (18) and well-documented information of the number of cases which may be missed in any single screening of population group are essential. Hence, newly found cases of carcinoma in situ after an interval of even two years are not necessarily related to incidence because evidence of its early transformation most likely *antedates the first examination by many* years.

Similar considerations are required for evaluation of prospective studies of untreated patients. The reported low progression rates of carcinoma in situ to invasive carcinoma[illegible] with the highest rate of 48 3 per cent[illegible] have led to the assumption that *either not all carcinomas* in situ have the same malignant potential or that different criteria were utilized for their subjective interpretation. Whatever the reasons may be, the results have failed to establish that carcinoma in situ by various past definitions consistently leads to invasive carcinoma. It is *therefore not certain as to how much validity may* be attributed to the malignant potential of dysplasias which was determined from their observation to the endpoint of carcinoma in situ only. Moreover the reported progression rates to carcinoma in situ in 1818 of 1922 cases (Table VI) ranged from 1 12 per cent to 16 0 per cent *after observation periods of* two months to 11 years[illegible]. In the remaining 104 cases, the progression to carcinoma in situ occurred at the rates of 42.3 per cent after three years (34) and of 50.0 per cent after six years (73). Observation of 350 of these patients for periods up to 10 years led to diagnoses of invasive carcinoma in 0 4 per cent to 3 8 per cent of the cases[illegible] and to 14 1 per cent of 78 patients after six years observation (73). Furthermore, 20.2 per cent to 53.8 per cent of the 1922 dysplastic lesions reverted to normal either spontaneously or as result of biopsies while rates for persistence of the lesions ranged from 15.4 per cent to 58 2 per cent.

Interpretation of results from observation of untreated lesions and their comparative evaluation is met with many variables. The effect of biopsies on malignant transformation of intraepithelial lesions is not certain. It was suggested that biopsies may influence the course of intraepithelial alterations.[illegible] However others were *unable to determine that biopsies resulted in* regression[illegible]. Truly undisturbed lesions probably do not exist. In addition to the problematic effects of biopsies, other factors may influence the course of the lesions such as hygiene, frequency of coitus, drugs, mode of contraceptives, infections,[illegible] and others. To eliminate the possible effects of biopsies intraepithelial neoplasias were observed by means of cytology alone or together with colposcopy [illegible] This obviously avoids any interference with the development of the lesion, but has the diagnostic disadvantage for assessment of the initial histologic alteration.

Furthermore the uncertainties of intraepithelial histologic interpretations must necessarily re flect in cytologic diagnoses because the basis for cytodiagnostic evaluations was established from comparative studies with histopathologic findings. In addition, incomplete representation in smears of all cells of the cervical lesion may lead to erroneous findings just as incomplete biopsies do not permit evaluation of the entire lesion.

Discrepancies in reported progression rates may have resulted from subjective interpretations by different criteria for cytologic and histopathologic diagnoses. Reclassification of tissue sections from initial biopsies according to the parameters of nuclear structures, cell alterations and polarity led in 33 cases to a close correlation of initial intraepithelial changes with their subsequent behavior (Table V). In the study of benign dysplasias the progression to incipient carcinoma in situ in two out of 11 cases with disorderly polarity against none of the seven with orderly polarity is insufficient for any valid conclusion. However those with initially incipient carcinoma in situ progressed to either carcinoma in situ or invasive carcinoma and all cases interpreted as carcinoma in situ progressed to invasive carcinoma.

Classification of Intraepithelial Uterine Cervix Alterations

The consistent relationship of altered cells in tissues to the histologic pattern of invasive carcinoma and to those of benign tissues from other sites and their comparative evaluation with cell in cervical intraepithelial lesions led to re-evaluation of existing concepts, criteria and classification. Based upon the results from this study it appeared incongruous that intraepithelial malignant neoplasia may proceed without prior malignant cell alterations and that malignant tumor may consist entirely of benign cell. Conversely, the presence of malignant tumor cells is not conceivable in benign tissues. Cellular malignant neoplastic changes may however occur in benign appearing histologic pattern as a result of early local or of advanced carcinogenic effects on areas adjacent to evident malignant epithelial alteration. Awareness of these basic biologic principles may often aid in the differentiation of intraepithelial lesions according to their benign or malignant potential. Reports that similar appearing histologic patterns may have different malignant potentials, indicates the limited value of any suggested terminologic classification without adequate definition of the morphologic aspects which invariably differ according to the biologic properties of intraepithelial alterations.

The inability to classify intraepithelial alterations of the uterine cervix in terms of prognosis led to attempts by the International Committee on Histologic Terminology to define carcinoma in situ and dysplasia.[11] Carcinoma in situ was described as "a surface epithelium in which throughout the whole thickness no differentiation takes place. The process may involve the cervical glands without thereby creating a new group. It is recognized that the cells of the uppermost layers may show some slight flattening. The very rare case of an otherwise characteristic carcinoma in situ which shows a greater degree of differentiation belongs to the exceptions for which no classification can provide" and dysplasia by all other disturbances of differentiation in the squamous epithelial lining the glands or covering of the surface."

Lack of epithelial differentiation occurs as a result of impaired or arrested cell maturation and may be found in a variety of benign conditions such as in cases of basal cell proliferation. Loss of differentiation may be found also in cases of subcylindrical cell or spindle cell proliferation in the endocervical canal and glands. Often squamous metaplasia and abnormal cellular changes may be present as a result of inflammation. Hence complete loss of differentiation may be entirely benign however such cases fit the classification of carcinoma in situ when defined on the basis of differentiation without consideration of cell structures.

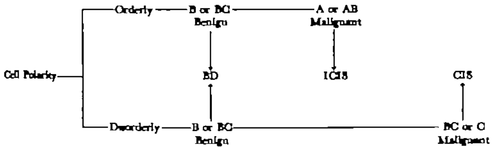
Furthermore the surface epithelium without differentiation may consist of cells which are arranged in an orderly manner with their cytoplasmic and nuclear long axis in uniform polarity or in a disorderly manner with lack of uniform polarity. In both cases, the epithelium may be benign or potentially malignant, depending upon the type of cellular alteration.

CONCLUSIONS

Criteria for identification of cervical intraepithelial lesions by combined evaluation of cell and tissue changes could not be correlated with previous classifications. Reclassification of cervical lesions by the evolved criteria was made with emphasis on the definition of intraepithelial alterations rather than on the suggested nomenclature for their designation.

Tissues with loss of cervical intraepithelial differentiation with either uniform or disorderly altered cell polarity either throughout or in the major part of the squamous epithelium, the canal or glands were classified in the presence of malignant alterations as intraepithelial malignant neoplasias and those with benign nuclear structures as benign dysplasias. The intraepithelial malignant neoplasias with uniform cell polarity were designated as incipient carcinoma in situ" and those with disorderly polarity as "carcinoma in situ (Table VII)

TABLE VII. *Intraepithelial Polarity and Structure of Cells*



"Carcinoma in situ" was defined as undifferentiated epithelium of the portio vaginalis, canal or glands in which cells with disorderly polarity had malignant nuclear changes with usually BC and C chromatin structures and high N/C ratios.

"Incipient carcinoma in situ" was defined as undifferentiated epithelium of the portio vaginalis, canal or glands in which cells with abnormal but uniform polarity had a high N C ratio and the malignant nuclear structures A or transitional forms AB and less frequently structure BC and C

The term "benign dysplasia" was applied to undifferentiated epithelium of the portio vaginalis, canal or glands in which the cells with either uniform or disorderly polarity had low or increased N/C ratio but benign nuclear structures and occasionally homogeneous pale or pyknotic nuclei This category included basal spindle cell proliferation in the squamous epithelial lining the canal or glands with or without squamous metaplasia or surface differentiation, subcylindrical cell proliferation with or without squamous metaplasia and parabasal cell proliferation in the squamous epithelium with or without the effects of acute or chronic inflammation

Thus, the examination at high microscopic magnifications of cell structures in undifferentiated epithelia with disorderly cell polarity permitted distinction of benign dysplasias from carcinoma in situ Similarly undifferentiated epithelia ith uniform cell polarity were identified by their cellular changes as either benign or incipient carcinoma situ and distinguished from carcinoma in situ

A prognostic diagnosis that carcinoma may not eventually develop in the uterine cervix with benign dysplasia should be made cautiously Similarly no prediction can be made that dysplastic and malignant transformation may never mature in normal cervical tissue

SUMMARY

The role of dysplasias determined from their development to carcinoma in situ is of doubtful validity by present definitions because 81 per cent to 98 9 per cent of dysplasias failed to progress to carcinoma in situ and 51 7 per cent t 86 6 per cent of alterations interpreted as carcinoma in situ did not develop invasive carcinoma The inability to define the lesions in prognostic terms led to comparative study of several parameters for identification of intraepithelial changes according to their malignant potential

Cells in tissue sections were studied t the highest magnification of the light microscope and the pathologic interpretation of their changes was determined from the histologic pattern of the same area by low power objectives Previously described nuclear structures A, B, C and their combined forms AB and BC were identified and their N C ratio, degree of chromasia and nucleolar b-normalities were evaluated Degrees of chromasia in the uclei with structure A were determined from the curved chromatin bands and small chromocenters around pale circular areas and in structure C from the larger chromocenters and their band ttachments, whereas nucleolar changes were examined in cells with structure B

I 111 benign tissues of various sites, the cells had either low or high N/C ratios and small or often large nuclei with pale chromatin in structure A, AB, BC and C and absence of abnormal nucleolar changes in structure B

In 39 invasive carcinomas, the cells had high N C ratio with hyperchromasia of uclear structures A, AB, BC and C and nucleoli of either increased size or w th abnormal nucleolar uclear ratios in those sociated with structure B

I 48 of 90 cervical tissue sections with loss of intraepithelial differentiation, the cells were comparable to those of 111 benign tissues of other sites and were therefore classified as Benign Dysplasias The remaining 42 cases had cells with the same alterations as those of 39 invasive carcinomas and were therefore designated as "intraepithelial malignant neoplasias

Study of cellular changes in relation to their nuclear structure and polarity did not shed further light on benign dysplasias which consisted of 29 cases with uniform and 19 with disorderly polarity. In contrast, the 42 malignant neoplasias revealed a close relationship between nuclear structures and polarity. In 21 cases, the association of malignant nuclear structures A or AB and rarely BC or C with uniform polarity led to their classification as "incipient carcinoma in situ" which differed from "carcinoma in situ" in which cells in disorderly polarity had predominantly malignant structures BC or C.

Study of mitoses revealed mitotic figures in 76 19 per cent of tissues with intraepithelial malignant neoplasias against 10 53 per cent of the benign dysplasias with uniform cell polarity and 41 38 per cent of those with disorderly polarity. In the malignant neoplasias with incipient carcinoma in situ and carcinoma in situ the chromatin structure of dividing cells in anaphase and telophase resembled those of invasive carcinoma. This finding rendered additional evidence for classification of both intraepithelial alterations as intraepithelial malignant neoplasias, whereas their dissimilar basic nuclear structures and cell polarity were interpreted to reflect different stages of malignant tumor development.

On the basis of these criteria, 33 initial tissue sections from a previously conducted 12 year study of untreated patients were coded and re-evaluated. Those classified as carcinoma in situ revealed transformation to invasive carcinoma after an average of less than 4 years. Cases with incipient carcinoma in situ developed either carcinoma in situ or invasive carcinoma after periods of 4 5 to 9 years whereas in 11 11 per cent of the benign dysplasias, incipient carcinoma in situ were found after a period of 7 5 years.

A consistent relationship between cell pathology and the histologic pattern was evident from lack of malignant cells in benign tissues of various sites, and presence of malignant neoplastic structures in cells of invasive carcinoma. It is therefore not feasible to interpret intraepithelial alterations with benign cells as carcinoma in situ and to render a benign diagnosis of intraepithelial alterations in presence of malignant neoplastic cells.

These observations led to the following definition of intraepithelial alterations which may originate in the cervical canal, the gland or in the squamous epithelial lining.

Carcinoma in Situ is defined as intraepithelial loss of differentiation with disorderly polarity of cells which have increased N/C ratios and hyperchromasia of nuclear structures BC or C often with abnormal mitoses in late anaphase telophase and metaphase.

Incipient Carcinoma in Situ denotes intraepithelial loss of differentiation in which cells in uniform polarity have increased N/C ratios and hyperchromasia of nuclear structures A or AB and rarely BC or C, frequently with abnormal mitoses in late anaphase and telophase.

Benign Dysplasias include all other intraepithelial alterations with loss of differentiation with either uniform or disorderly polarity of cells which lack any nuclear evidence of a potential for malignant neoplastic transformation.

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A Test Statistic for Detecting the Presence of Abnormal Cells in a Sample

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Considerable research effort is presently directed toward development of prescreening methods. These should be capable of examining, at high speed, very large number of cells from patient, to declare the sample either to be completely normal, or to require further examination In real world situation, samples consist of cells of different types, all of which vary with respect to their properties. One would, therefore, be dealing with mixture of distributions which will usually have several modes. Moreover most attempts t extracting information from the sample are based on measurements of several characteristics for each cell. The sample, therefore, comes from multivariate, multimodal distribution

The specific difficulty of the prescreening problem lies in the fact that the abnormal" sample may contain but very small percentage of abnormal cells. These hav to be detected against the background of an overwhelming majority of cells as found in any normal sample as well It is the purpose of this paper to describe test statistic on the basis of which the hypothesis of "no abnormality" can be accepted or rejected The proposed statistic lends itself to efficient computational evaluation.

We shall discuss this statistic in terms of single cell characteristic The random variable x, however could represent linear combination of the measurements of various cell characteristics, formed to yield optimum discrimination We shall also assume that the two cell populations, normal (I) and abnormal cells (II) both follow Gaussian distributions with different means and variances. By simple transformation, the problem can always be brought into form in which the normal cell population is represented by Gaussian distribution with mean 0 and variance 1 and the abnormal cell population by another Gaussian distribution with some mean μ and some variance σ^2 Let t now be assumed that individual cells from cell population stemming from patient with unknown case history are observed Values x_n are observed for the variable in cells 1 2, 3 n.

This collection of cells may now represent either population consisting only of cell population I they are all normal cells, or mixture of I and II, i normal cells, plus certain percentage of abnormal cells If the proportion of cells from the abnormal population (II) is denoted by Θ then the mixture has the ratio $1-\Theta$ Θ of normal to abnormal cells. We shall call Θ the mixing parameter

μ and σ^2 are known from observation of sets of individual tumor cells of confirmed diagnosis I all types of interest, $\sigma^2 > 1$ and we shall restrict our attention to this case The mixing parameter Θ is not known for particular clinical sample, but threshold $\lambda > 0$ could be specified In this way we are led to the following set-up

The observed characteristic has density function which for mixing parameter Θ is given by

$$f_\Theta(x) = (1-\Theta)\frac{1}{\sqrt{2\pi}}e^{-x^2/2} + \Theta\frac{1}{\sqrt{2\pi}\,\sigma}e^{-(x-\mu)^2/2\sigma^2}\,dx \tag{1}$$

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in which the first term denotes the contribution from the normal cells and the second term that from the abnormal cells. The unknown parameter Θ always lies between 0 and 1. The case $\Theta = 0$ corresponds to a cell population with no abnormal cells in it, and as Θ increases to 1 a higher and higher percentage of abnormal cells enters into the population.

Examples of the type of density function which would for the assumed model be recorded on a prescreening device are shown in Figure 1a and 1b. Visual inspection of such profiles certainly could detect small admixtures of abnormal cells, even down to 1 per cent—provided that the means difference between normal and abnormal cell populations is substantial. This is shown in Figure 1a. The large peak on the left represents the normal cells after transformation to zero mean and unit variance. The peak toward high values represents the abnormal cell population. The mixing parameter Θ which determines the percentage of admixed abnormal cells in the sample, was assumed to have values of 0.01, 0.10, 0.20 and 0.40. The profiles have the following parameters:

	Mean	Standard Deviation
Normal Cells	$\mu = 3.0$	$\sigma = 0.45$
Abnormal Cells	$\mu_1 = 12.0$	$\sigma_1 = 0.90$
Transformed variables	$\mu = (\mu_1 - \mu)/\sigma$	$\sigma = \sigma_1/\sigma$
Normal Cells	0.0	1.0
Abnormal Cell	$\mu = 4.5$	$\sigma = 2.0$

As soon, though, as the mean difference between normal and abnormal cell populations is smaller, visual inspection renders hardly any clues for the detection of the presence of a small admixture of abnormal cells, as can be seen in Figure 1b. The following parameters define these profiles:

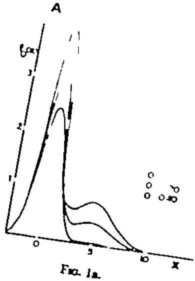

Fig. 1a.

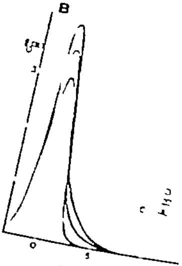

Fig. 1b.

Normal Cells	$\mu = 3.0$	$\sigma = 0.45$
Abnormal Cell	$\mu_1 = 6.0$	$\sigma_1 = 0.90$
Transformed variables		
Normal Cells	0.0	1.0
Abnormal Cells	$\mu = 1.5$	$\sigma = 0$

Again, four curves, for values of 0.01, 0.10, 0.20 and 0.40 of the mixing parameter are shown.

To be clinically useful, a prescreening device must comprise provisions for an objective test of clinical samples normalcy. This test must be based on appropriate, and efficient statistical procedures.

Under the model given by (1), we shall test the hypothesis H_0 $\Theta = 0$ against H_1 $\Theta = \lambda$ on the basis of observations $x_1, \ldots, x_n$ where λ is the threshold value mentioned above. However for the purpose of diagnosis, acceptance of H_0 $\Theta = 0$, or H_1 $\Theta = \lambda$ may well be interpreted as acceptance of H_0 $\Theta < \lambda$ or H_1 $\Theta > \lambda$ respectively.

By a well known result in the general theory of hypothesis testing, the best test for H_0 against H_1 at a given level of significance α is the one by which the hypothesis H_0 is accepted when

$$\prod_{1} \frac{f_1(x_i)}{f_0(x_i)} < B \tag{2a}$$

and rejected in favor of H_1 when

$$\prod_{1} \frac{f_1(x_i)}{f_0(x_i)} \geq B \tag{2b}$$

where the constant B is so chosen that the test operates at a given level of significance α, i.e. rejects the hypothesis wrongly with probability α. The test given by (2) is known as the likelihood ratio test. The use of a product of likelihood ratios unwieldy but it can be easily reduced to an equivalent criterion involving sums by the logarithmic transformation. In this equivalent version the hypothesis H_0 is accepted when

$$\sum_1 \log \frac{f_1(x_i)}{f_0(x_i)} < A \tag{3a}$$

and rejected in favor of H when

$$\sum_1 \log \frac{f_1(x_i)}{f_0(x_i)} \geq A \tag{3b}$$

here the constant A in (3) is related to the constant B in (2) by the relation A = log B. There is, however an additional reason for preferring the criterion given in (3) which is related to the approximate distribution of the test statistic when the sample size is large, as discussed in the Appendix.

It should be kept in mind that throughout this paper all logarithms are to the base e. The sum of the logarithms of all likelihood ratios can then be written as

$$\sum_1 \log \frac{f_1(x_i)}{f_0(x_i)} = \log (1 - \lambda) + \log \left[1 + \frac{\lambda}{1 - \lambda}\varphi(x_i)\right] \tag{4}$$

where

$$\varphi(\;) \quad \text{[illegible]} \tag{5}$$

It is evident that for clinical cell sample consisting of roughly 100,000 cells it would be very difficult to compute these expressions for every cell. Furthermore due to the complicated nature of the expression

$$\log \left[1 + \frac{\lambda}{1 - \lambda}\varphi(\;)\right]$$

it becomes ery difficult to derive the approximate distribution of the test statistic under different values of Θ when is large. In an effort to develop test statistic which reduces both of these difficulties considerably the following approach is taken. For certain range of observations the expression $\lambda\varphi(x_i)$ $(1 - \lambda)$ assumes very small value, so that the contribution of these observed values to the sum of log likelihoods in (4) is negligible. For these observations the contribution to the test statistic can simply be taken as log $(1 - \lambda)$. I computational terms this means that instead of the numerical values of such their count multiplied by an appropriate factor goes into the making of the test statistic. Now equation (4) can also be written as

$$\sum_1 \log \frac{f_1(x_i)}{f_0(\;)} = \log \lambda + \sum_1 \log \varphi(x_i) + \sum_1 \log \left[1 + \frac{1 - \lambda}{\lambda}\frac{1}{\varphi(x_i)}\right] \tag{6}$$

and there is range of x for which the expression $(1 - \lambda)/\lambda\varphi(\;)$ is very small. For these observations one may take as their contributions to the test statistic the values $\log \lambda + \log \varphi(\;)$ which is much easier to compute.

The ranges of for which neither of the expressions

$$\frac{\lambda}{1 - \lambda}\varphi(x_i) \quad \text{or} \quad \frac{1 - \lambda}{\lambda}\frac{1}{\varphi(x_i)} \tag{7}$$

is very small have to be treated differently. W replace the contributions of the individual in this category by common contribution, which is in some sense representative of the various contributions coming from this category. The effect of this averaging will depend on the choice of the veraging formula, the spread of the range of these and the probability distribution over this range for $\Theta = 0$, and $\Theta = \lambda$.

Two questions arise with the definition of these ranges. The first is what is to be considered to be small value so that the appropriate treatments suggested above may be considered justified, and the second is what is to be done with those values of for which neither of the two expressions I (7) can be considered to be small. The situation is shown schematically in Figure 2, where these ranges are indicated.

These ranges are symmetric with respect to the point $-\mu/(\sigma^2 - 1)$ on the axis. Small is defined as having value less than The positions of A B B and A thus depend on ϵ, and

$$B_1 \leq \quad \leq B_2 \Leftrightarrow \frac{\lambda}{1 - \lambda}\varphi(x_i) \leq$$

$$\left.\begin{matrix} \leq A_1 \\ \text{or} \\ \geq A_2 \end{matrix}\right\} \Leftrightarrow \frac{1 - \lambda}{\lambda}\frac{1}{\varphi(x_i)} \leq \epsilon$$

When is given certain fixed value and both the difference of means and the ratio of variances are known for the normal cells (I) and abnormal cells (II) the constants A A B B_2 which determine these ranges are known. Formulas for these constants in terms of the parameters μ and σ the

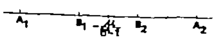

FIG. 2.

criterion for smallness ϵ and the threshold value λ are given as

$$A_1 = -\frac{\mu}{\sigma^2-1} - \left[\frac{2\sigma^2}{\sigma^2-1}\log\left(\frac{(1-\lambda)\sigma}{\lambda\epsilon}\right) + \frac{\mu^2\sigma^2}{(\sigma^2-1)}\right]^{1/2}$$

$$A_2 = -\frac{\mu}{\sigma^2-1} + \left[\frac{2\sigma^2}{\sigma^2-1}\log\left(\frac{(1-\lambda)\sigma}{\lambda\epsilon}\right) + \frac{\mu^2\sigma^2}{(\sigma^2-1)}\right]^{1/2}$$

$$B_1 = -\frac{\mu}{\sigma^2-1} - \left[\frac{2\sigma^2}{\sigma^2-1}\log\left(\frac{(1-\lambda)\sigma}{\lambda}\right) + \frac{\mu^2\sigma^2}{(\sigma^2-1)}\right]^{1/2} \qquad (8)$$

$$B_2 = -\frac{\mu}{\sigma^2-1} + \left[\frac{2\sigma^2}{\sigma^2-1}\log\left(\frac{(1-\lambda)\sigma}{\lambda}\right) + \frac{\mu^2\sigma^2}{(\ \ -1)^2}\right]^{1/2}$$

We shall choose ϵ in the interval

$$\frac{\lambda}{1-\lambda}\frac{1}{\sigma}e^{-\mu} \quad {}^{-n} < \quad < 1$$

so that the constants A_1, A_2, B_1, B_2 satisfy the inequality

$$A_1 < B_1 < B_2 < A_2$$

as in Figure 2

Two kinds of approximation are involved in the proposed test statistic. The first kind arises when the quantity

$$\log\left[1 + \frac{\lambda}{1-\lambda}\varphi(\ \ )\right]$$

is ignored for an observation x_i lying in the interval (B_1, B_2) or the quantity

$$\log\left[1 + \frac{1-\lambda}{\lambda}\frac{1}{\varphi(x_i)}\right]$$

is ignored for an observation x_i which is either less than A_1 or greater than A_2. The second kind of approximation arises when for an observation x_i lying in one of the intervals (A_1, B_1) or (B_2, A_2) the quantity

$$\log\left[1 + \frac{\lambda}{1-\lambda}\varphi(x_i)\right]$$

is replaced by an average.

For a very small ϵ the first type of approximation is good but the effect of the second type of approximation becomes pronounced. As ϵ becomes larger and larger the first type of approximation becomes worse and worse but the effect of the second type of approximation diminishes. The value of ϵ is therefore to be chosen in such a way as to keep a balance between these two types of approximations in order to achieve maximum power for the test procedure.

We now look at the question of how to treat the observations x_i in the intervals (A_1, B_1) and (B_2, A_2) for which neither of the two quantities in (7) is smaller than ϵ. If these intervals were very narrow, as would be the case when ϵ is moderately large, replacing $\varphi(x_i)$ for such x in the expression

$$\log(1-\lambda) + \log\left[1 + \frac{\lambda}{1-\lambda}\varphi(x_i)\right]$$

by an average for each interval would be justified in view of the fact that $\varphi(x_i)$ is a smooth function of x. Furthermore, due to the symmetry of the intervals under consideration, the same average can be used for both intervals. As to the appropriate selection of the averaging formula, we may use for all x in (A_1, B_1) or (B_2, A_2)

$$\varphi_1(x\ ) = \varphi\left(\frac{A_1 + B_1}{\ }\right)$$

or

$$\varphi_2(x\ ) = \frac{(\varphi(A_1) + \varphi(B_1))}{\ }$$

or

$$\varphi_3(x_i) = \frac{\varphi(u_1) + \quad + \varphi(u_k))}{k}$$

where u_1 u_k are selected points in the interval $(A\ \ B\ )$ and k is large, say 10, or

$$\varphi\ (x_i) = \frac{w_1\varphi(u_1) + \quad + w_k\varphi(u_k)}{w\ + \quad + w}$$

where u u are as above and the weights w are the densities at u under the Null hypothesis, i.e. $w\ = f_0(u\ )$. In this paper we have investigated the efficacies of the averaging formulas and φ of which is found to be superior in all situations considered.

In general, with an averaging formula $\hat{\varphi}$ (of which φ φ described above are special cases) the original likelihood test statistic

$$T = \sum_1 \log\frac{f_1(x_i)}{f_0(x_i)}$$

is approximated by this approach as

$$\hat{T} = n\log(1-\lambda) + C_1n_1 + C_2n_2 + C_3\sum\left(x_i + \frac{\mu}{\sigma^2-1}\right) \qquad (9)$$

---

here

$$C_1 = \log\left[1 + \frac{\lambda}{1-\lambda}\hat{\phi}\right]$$

$$C_2 = \log\left(\frac{\lambda}{(1-\lambda)\sigma}\right) - \frac{\mu^2}{2(\sigma^2-1)} \qquad (10)$$

$$_nC_3 = \left(1 - \frac{1}{\sigma^2}\right)\Big/ 2$$

= number of in (A B₁) U (B A₂) n = number of in (− ∞ A] U [A ∞) $\sum$ denotes the sum over only those which are in (− A] U [A ∞) and $\hat{\phi}$ is the representative value of $\varphi(x_i)$ for all x in (A B₁) U (B₂ A₂)

The practical computational procedure thus involves the following steps

Step 1 Transformation of original parameters and observations Let μ_0 be the mean and standard deviation respectively of the normal cell population (I) and μ σ the mean and standard deviation respectively for the abnormal cell population (II) in given situation Let y y be the observed values of the cell characteristic for cells 1 n We transform

$$x_1 \quad \frac{y - \mu_0}{\quad} \qquad = \frac{y - \mu_0}{\quad} \qquad x_n = \frac{y - \mu_0}{\quad}$$

For these transformed observations, the normal cell population (I) has mean 0 and standard deviation 1 and the abnormal cell population (II) has mean $\mu = (\mu - \mu_0)$ and standard deviation 1/ σ must be greater than 1 for the validity of what follows

Step 2 Choice of and determination of A A B B₂ This is discussed in the Appendix here for various parametric combinations suggested values of and the resulting values of A A B B according to formula (8) are given in Table II

Step 3. Summarization of data The entire range of observations is divided into three sets

$$S = (A \quad B\,) \cup (B \quad A_2)$$

$$S = (- \quad A\,] \cup [A \qquad)$$

$$S = [B \quad B\,]$$

The transformed observations x_n are now processed to obtain

= number of observations in S
= number of observations in S
= number of observations in S
$\sum$ = sum of observations in S
$\sum$ x^2 = sum of squares of observations in S

Then, from $\sum$ and $\sum$ x^2 we compute = + + = total number of observations, and

$$\sum\left(\quad + \frac{}{\sigma^2-1}\right) = \sum x_i + \frac{2\mu}{\sigma^2-1}$$

$$\sum x_i + n_3\left(\frac{\mu}{\sigma^2-1}\right)^2$$

Step 4 Choice of the averaging formula $\hat{\phi}$ and determination of C C C In this paper we have studied two formulas for $\hat{\phi}$ viz φ and φ given above, of which φ is found to be superior Of the constants C C C given by (10) only C depends on the choice of $\hat{\phi}$ for which we have used the formula for φ C C C are given in the Appendix in Table III for the suggested value of and for $\hat{\phi} = \varphi((A + B\,)/2)$ corresponding to various parametric combinations.

Step 5 Computation of approximate test statistic We now compute T by inserting the summaries n, and $\sum(\quad + \mu/(\quad - 1))^2$ obtained in Step 3, and the constants C C C obtained in Step 4 into formula (9)

Step 6 Normalization of $\hat{T}$ We normalize $\hat{T}$ by computing

$$T = \frac{T - \quad \log(1-\lambda) - nm(\;)}{\sqrt{nv(\;)}}$$

where m( ) is the mean and ( ) is the variance of $\hat{T}$ /n under the Null hypothesis. Formulas for m( ) and ( ) and tables for m( ) and v( ) corresponding to various parametric combinations are given in the Appendix (Table III)

Step 7 Test procedure Accept H_0 if T < $\Phi^{-1}(1-\alpha)$ and reject H_0 in favor of H if T $\geq$ $\Phi^{-}(1-\alpha)$ where $\Phi^{-1}(1-\alpha)$ is the upper $100(1-\alpha)$ per cent point of the Gaussian distribution with mean 0 and variance 1 In particular

$$\text{for } \alpha = 0\,001 \qquad \Phi^{-1}(1-\alpha) = 3\,090$$

$$\text{for } \alpha = 0\,01 \qquad \Phi^{-1}(1-\alpha) = 2.327$$

and

$$\text{for } \alpha = 0\,05 \qquad \Phi^{-1}(1-\alpha) = 1\,645.$$

Power of the above test procedure, which we refer to as Test 1 1 probability of rejecting the Null hypothesis correctly by this test is discussed in the Appendix, and powers for some selected values of the sample size are given in Tables IVa, IVb and IVc.

In the Appendix we also discuss briefly another test known as the locally most powerful test of H_0 against H Since this test can be very bad in certain situations we have not studied it in any great detail in this paper We shall refer to this test as Test 2

DISCUSSION

Developments in the technology of cell sorting and high speed flow-through devices has produced several prototypes of instruments for which application in the area of prescreening for cervical cancer is being discussed.[3-6, 8, 9] Some of these devices display the result in the form of two-dimensional histograms on the CRT, others permit a representation as one-dimensional histograms. To the best of the authors' knowledge, none of these instruments employs, at the present time, a recognition logic of one sort or another which determines a criterion on the basis of which the result of the sample's examination is classified. Yet to become clinically useful, decisions about the samples' normalcy must be a machine function, and will undoubtedly be incorporated sooner or later.

The test statistic described here is suggested as the basis for such decisions. Not only can it be evaluated on line with relatively moderate computational effort, it is also, primarily due to the large sample sizes and nature of the test employed, *extremely sensitive.*

For a certain average parametric combination, let the desired level of significance for the test of the Null-hypothesis be set to 0.001. This means a false alarm rate of one per 999 samples. Furthermore, let a sample size of 200 000 be assumed, and an admixture of but 200 abnormal cells. The power of the test then amounts to .99, i.e., one would face a probability of 1 per cent missed positives. If one *allows the false alarm rate to be higher,* as one would in view of the inequality of risk involved between a false alarm, or a missed positive, say at 5 per cent, the power of the test becomes 0.999, i.e., a missed positive rate of one in 999 can be attained.

It should be pointed out that a real cell sample may not follow the underlying mathematical model quite so closely that the very high levels of decision certainty can be maintained. On the other hand, relatively simple transformations of the measured random variables could result in a high degree of fit.

However, the power and usefulness of this test statistic should be seen against the error rates presently under discussion for prescreening devices,[2, 8] where missed positive rates of several per cent, and false alarm rates of up to 30 per cent are considered *acceptable,* even though out of necessity. Inspection of the range of parametric combinations will show that most conservative assumptions about the increase of mean and variance in abnormal cell populations were made. For abnormal cell populations with substantially increased parameter values, the sensitivity of the test becomes even greater.

APPENDIX

Test 1. An Approximation to the Most Powerful Test

We shall first obtain the approximate distribution of T when the mixing parameter has the value Θ. Then $\Theta = 0$ will correspond to the situation under the Null hypothesis H_0 and $\Theta = \lambda$ will correspond to the situation under the alternative hypothesis H_1. Throughout, Φ stands for the *cumulative distribution function*

$$\Phi(x) = \int_{-\infty}^{x} \frac{1}{\sqrt{2\pi}} e^{-t^2/2}\, dt$$

Define

$$A_1 = \frac{A_1 - \mu}{\sigma} \quad B_1 = \frac{B_1 - \mu}{\sigma}$$

$$A_2 = \frac{A_2 - \mu}{\sigma} \quad B_2 = \frac{B_2 - \mu}{\sigma}$$

where A_1, A_2, B_1, B_2 are given in (8), and

$$l = -\frac{\mu}{\sigma^2 - 1} \quad k = \frac{k - \mu}{\sigma}$$

Let $g_1(x)$ and $g_2(x)$ be defined as

$$g_1(x) = 1 \text{ if } x \text{ is in } S_1$$
$$= 0 \text{ if } x \text{ is not in } S_1$$

and

$$g_2(x) = 1 \text{ if } x \text{ is in } S_2$$
$$= 0 \text{ if } x \text{ is not in } S_2$$

where the sets S_1 and S_2 are defined in (11). Then we can rewrite formula (9) as

$$T = n \log (1 - \lambda) + \sum_{1}^{n} [C_1 g_1(x_i) + C_2 g_2(x_i) + C_3 g_2(x_i)(x_i - k)^2] \tag{12}$$

The approximate distribution of T for large n can now be derived from the Central Limit Theorem of probability theory (see Chung, Theorem 6.4.4) because in formula (12) T is expressed as the sum of independent random variables. This was one of our reasons for taking the logarithms of the likelihood ratio in the first place. From the Central Limit Theorem we concluded that for large n, T is approximately Gaussian with mean $n\{\log (1 - \lambda) + m(\Theta)\}$ where $m(\Theta) = E_\Theta[C_1 g_1(x) + C_2 g_2(x) + C_3 g_2(x)(x - k)^2]$ and variance $nv(\Theta)$, where $v(\Theta) = \mathrm{var}_\Theta[C_1 g_1(x) + C_2 g_2(x) + C_3 g_2(x)(x - k)^2]$, where E_Θ and var_Θ denote the mean and the variance respectively of a random variable with density function $f_\Theta(x)$ given in (1). We shall now obtain this expectation and this variance Define

$$P \quad \Phi(B_1) - \Phi(A_1) + \Phi(A_2) - \Phi(B_2)$$

$$P' = \Phi(B_1') - \Phi(A_1') + \Phi(A_2') - \Phi(B_2')$$

$$P = \Phi(A_1) + 1 - \Phi(A_2)$$

$$P' = \Phi(A_1') + 1 - \Phi(A_2')$$

$$f_1(\Theta) \quad (1-\Theta)P + \Theta P'$$

$$f_2(\Theta) \quad (1-\Theta)P + \Theta P'$$

$$f_3(\Theta) = (1-\Theta)\left[1 + k - \int (x-k)^2 \frac{1}{\sqrt{2}} e^{-x^2} \, dx\right] + \Theta\sigma^2\left[1 + k - \int^{d} (x-k')^2 \frac{1}{\sqrt{2\pi}} {}^{-x^2} \, dx\right]$$

Then

$$m(\Theta) = C_1 f_1(\Theta) + C_2 f_2(\Theta) + C_3 f_3(\Theta)$$

This gives the expectation T obtain the variance define

$$q_{11}(\Theta) \quad f_1(\Theta) \qquad q_{22}(\Theta) = f_2(\Theta)$$

$$q_{33}(\Theta) \quad (1 \quad \Theta)\left[3 + 6k + k^4 - \int (x \quad k)^4 \frac{1}{\sqrt{2\pi}} e^{-x^2} \, dx\right] + \Theta\sigma^4\left[3 + 6k + k - \int (- k)^4 \frac{1}{\sqrt{2}} {}^{-x^2} \, dx\right],$$

$$q_{12}(\Theta) = q_{21}(\Theta) = q_{23}(\Theta) = q_{32}(\Theta) = 0,$$

and

$$q_{13}(\Theta) = q_{31}(\Theta) = f(\Theta)$$

and let

$$(\Theta) \quad q(\Theta) - f(\Theta)f(\Theta) \qquad i, j = 1\ 2, 3$$

Then

$$(\Theta) = C_1^2 v(\Theta) + C_2^2 v(\Theta) + C_3^2 v_{33}(\Theta) + 2C_1C_2 v(\Theta) + 2C_1C_3 v_{13}(\Theta) + 2C_2C_3 v_{23}(\Theta).$$

This gives the variance

Thus when is large and Θ is the mixing parameter $\hat{T}$ is approximately Gaussian with mean $n\{\log(1-\lambda) + m(\Theta)\}$ and variance (Θ) In particular under the Null hypothesis

$$T = \frac{\hat{T} - \log(1-\lambda) - nm(\ )}{\sqrt{nv(\ )}}$$

is approximately Gaussian with mean 0 and variance 1 This justifies the last two steps in the description of Test 1

For large n, and for mixing parameter Θ the power of Test 1 is approximately

$$\pi(\Theta) = \Pr_\Theta[T \geq \Phi^{-1}(1 - \ )] \simeq 1 - \Phi\left[\frac{\sqrt{v(\ )}\Phi^{-1}(1-\alpha) - \sqrt{\ }\{m(\Theta) - m(\ )\}}{\sqrt{v(\Theta)}}\right]$$

We shall now examine the effect of ϵ on Test 1 Let us fix our attention t particular parametric combination, viz $\mu = 0.6$, $\sigma = 2.5$ and $\lambda = 0.001$ and consider the case when the level of significance $\alpha = 0.05$, and the sample size is $= 100\,000$ For this situation the effect of on Test 1 is reflected in Table I The general pattern seen in this table is typical of all parametric combinations that we have studied

On the basis of Table I we arrive at the following conclusions

1) For very small values of practically all of the probability under the Null hypothesis is carried by the set S and for values of on the other end, practically all of the probability under the Null hypothesis is carried by the set S Since normal approximation to the binomial distribution is essentially involved here, w should avoid the situation where only one set carries practically all of the probability so that the normal approximation is reasonably good For this reason, w avoid the very low and the very high values of For sample rule of thumb concerning normal approximation to the binomial distribution, see Mendenhall.

2) Another reason for avoiding these extreme values of ϵ is that the matrix (((0))) becomes near singular in these situations giving rise to a loss in numerical accuracy Not that for these values of ϵ, ( ) is nearly zero

3) As increases, there is first sudden drop in the power of Test 1 and then t keeps steadily increasing This is because the set S_1 carries less and less probability as ϵ increases This is the set in which $\varphi(\ )$ for all observations is replaced by one representative value, and therefore with increasing ϵ the effect of this kind of pproximation diminishes. The initial drop in the power was due to the other approximation involved in neglecting one or the other of the quantities in (7) for observations in S_2 or S_3. Since the probability of S increases with increasing this approximation becomes worse and worse How-

TABLE I *Effect of [illegible] on Trial 1* (μ = 0.6 σ = 2.5 λ = 0 001 α = 0 05 n = 100 000)

	A	B	B	A₁	P( )	P(S)	m(ε)	(ε)	Power
000 9869	−6 3	−0 13	−0 10	6 00	08	01	01962	00001	8[illegible]
0007738	−6 09	−1 40	1 17	5 87	70	710	01 00	00143	677
001 [illegible]74	−5 [illegible]	−2 09	1 86	5 67	043	31	00[illegible]74	00182	729
00[illegible]64	−5 68	− [illegible]	2 43	5 45	011	89	00373	00120	860
0181014	−5 43	−3 14	91	5 20	001	907	00120	00035	947
05[illegible]013	−5 17	−3 51	3 33	4 94	001	990	00033	00020	979
1701470	−4 80	−3 [illegible]	3 71	4 66	000	1 000	00010	00006	980

Note P(S) P(S) and P(S) denote the probability contents of the sets S S and S respectively under the Null hypothesis Since P(S_3) is 0 to three places of decimal in all cases in this table, we have omitted the column for P(S) Throughout ε = ε (A + B))

TABLE II *S[illegible] [illegible] and Boundaries of the Th[illegible] Regions*

λ				A	B	B	A₁
001	1 3	1 5	003	−8 17	−4 44	04	5 77
		5	003	−5 [illegible]	−2 84	27	5 35
		4 0	003	−5 [illegible]0	−2 66	46	5 30
	3 0	1 5	00	−10 04	−6 90	10	5 [illegible]4
		2 5	0003	−6 00	− 17	1 03	5 63
		4 0	0003	−6 08	−1 65	1 [illegible]3	5 68
	[illegible] 0	1 5	00003	−15 47	−11 3[illegible]	1 72	5 87
		5	00003	−8 [illegible]4	− 93	0 65	3 95
		4 0	000[illegible]0	−6 41	−2 41	1 61	5 64
01	1 5	1 5	010	−7 33	−3 36	0 96	4 93
		2 3	010	−5 2[illegible]	−1 92	1 35	4 67
		4 0	010	−4 87	−1 8[illegible]	1 66	4 67
	3 0	1 5	010	−9 29	−6 19	1 39	4 41
		2 5	010	−5 68	− 62	1 48	4 54
		4 0	010	−5 0[illegible]	− 09	1 69	4 6[illegible]
	6 0	1 5	0001	−14 [illegible]	−10 86	1 [illegible]6	5 35
		3	001	−7 29	−3 34	1 06	5 00
		4 0	001	−3 88	−1 16	0 [illegible]6	5 08
1	1 5	1 5	050	−6 01	− 35	0 15	3 64
		5	0[illegible]0	−4 17	−1 17	0 60	3 59
		4 0	050	−3 87	−1 [illegible]	1 09	3 67
	3 0	1 5	0[illegible]0	−8 13	−3 80	1 00	3 33
		2 5	050	−4 64	− 0[illegible]	0 93	3 50
		4 0	050	−4 01	−1 38	1 18	3 63
	6 0	1 5	0003	−14 [illegible]1	−10 6[illegible]	1 0[illegible]	4 61
		5	003	−6 45	− 86	0 58	4 17
		4 0	01	−4 88	−1 0[illegible]	0 2[illegible]	4 08

ever except in the initial part of the range of ε the diminishing effect of the averaging ε(x) over the set S_1 more than offsets the increasing effect of the latter approximation.

4) From these considerations it seems that the value of for which P(S) lies between 0 01 and 0 02 is desirable

5) The above comment on the relative magni-

TABLE III *Value f C* C_1 C_4 m( ) ( )

λ			C_1	C	C_4	m(n)	(n)
001	1 5	1 5	0 522	−8 21	0 278	00667	00210
		2 5	0 514	−8 04	0 420	00437	00136
		4 0	0 528	−8 37	0 469	00357	00116
	3 0	1 5	0 408	−10 91	0 278	00736	00293
		2 5	0 101	8 69	0 420	01672	00141
		4 0	0 0962	8 60	0 469	01480	00121
	6 0	1 5	0 264	−21 71	0 278	01129	00286
		2 5	0 0507	−11 25	0 420	01319	00049
		4 0	0 139	−9 49	0 469		
01	1 5	1 5	0 286	−5 90	0 278	04833	01149
		2 5	0 272	−5 73	0 420	03169	00766
		4 0	0 297	−6 0	0 469	02377	00634
	3 0	1 5	0 414	−8 60	0 278	03900	01295
		2 5	0 317	−6 37	0 420	02335	00697
		4 0	0 31	6 28	0 469	01987	00585
	6 0	1 5	0 272	−19 40	0 278	02840	00632
		2 5	0 179	8 34	0 420	02601	00598
		4 0	0 0710	−7 18	0 469	03410	00127
1	1 5	1 5	0 358	−3 50	0 278	15978	05283
		2 5	0 330	−3 33	0 420	13032	02747
		4 0	0 378	−3 66	0 469	08098	02719
	3 0	1 5	0 519	−6 20	0 278	08992	07566
		2	0 407	−3 97	0 420	07797	02765
		4 0	0 401	−3 88	0 469	07168	02485
	6 0	1 5	0 342	−17 00	0 278	05283	01540
		2 5	0 230	−6 54	0 420	06536	01120
		4 0	0 161	−4 78	0 469	09169	00686

Note These alues orrespond to the suggested alues of given in Table II and the eraging formula $\varphi((A_1 + B_1)/2)$

rades of two kinds of approximations was only for the averaging formula $\hat{\varphi} = \varphi((A_1 + B_1)/2)$ and for this $\hat{\varphi}$ w cannot choose the alue of which apparently gives rise to maximum power because for these the normal pproximation is poor and the matri (((0))) is near singular

6) Since the approximation due to averaging $\varphi($ $)$ the set S outweighs the other approximation strongly suspect that th better averaging formula, such as φ or φ the bov complications would not arise and the power of the test would be much improved.

7) The probability content of the set S_1 under the Null hypothesis is extremely small all cases hat w has studied For the alue of that w has suggested in Table II, this probability is also small und the alternative hypothesis. It is only for the observations in this set that w have to compute the sum, and the sum of squares. For the other sets only the counts are needed in order to compute the *test statistic* The small alues for the probability contents of S under the Null hypothesis and the alternative hypothesis is therefore desirable from the point of view of computational ease

W now give the suggested values of e, and the resulting values of A A B B C C C m( ) and v( ) and the powers of Test 1 for various parametric combinations, levels of significance, and sample sizes (T bles II III nd IV)

The suggested values of e hav been so chosen as to keep the probability under the Null hypothesis distributed over the two sets S and S Where the *Null* and the alternativ hypotheses are

TABLE IVa *Power of Test I* α = *001*

λ	μ		(In thousands) 25	50	100	200	400
001	1 5	1 5	005	008	015	034	090
		2 5	561	750	[illegible]	993	
		4 0	946	992			
	3 0	1 5	333	575	837	990	
		2 5	886	974	999		
		4 0	973	998			
	6 0	1 5	999				
		5	999				
		4 0	998				
01	1 5	1 5	461	829	993		

Note These powers correspond to the values given in Table II For all other combinations of λ = 001 .01 1; μ = 1.5 3.0 6.0 and σ = 1.5 2.5 4 0 the power differs from 1 by less than .0005

TABLE IVb *Power of Test I* α = *01*

λ	μ		(In thousands) 25	50	100	200	400
001	1.5	1 5	032	047	077	130	.275
		2 5	665	829	954	997	
		4 0	954	993			
	3 0	1 5	518	748	939	997	
		2.5	911	981	999		
		4 0	976	998			
	6 0	1 5	999				
		2 5	999				
		4 0	998				
01	1 5	1 5	710	946	999		

Note These powers correspond to the values of given in Table II For all other combinations of λ = .001 .01 1 μ = 1.5 3.0 6.0 and σ = 1.5 .5 4.0 the power differs from 1 by less than .0003

TABLE IVc *Power of Test I* α = *05*

λ			(In thousands) 25	50	100	200	400
001	1 5	1 5	119	156	.222	336	523
		5	749	883	973	999	
		4 0	960	995			
	3 0	1 5	682	863	976	999	
		5	929	986	999		
		4 0	979	998			
	6 0	1 5					
		5	999				
		4 0	998				
01	1 5	1 5	872	986			

Note These powers correspond to the values of given in Table II For all other combinations of λ = .001 .01 1 μ = 1.5 3 0 6.0 and σ = 1.5 2.5 4.0 the power differs from 1 by less than .0005 far apart, the power is high for most reasonable choices of ϵ, and in these cases we have been able to maintain the probability of S higher than 10 or 15 per cent. In certain cases, however we had to be more careful about the power In these cases we have kept the probability of S between 1 and 2 per cent. It is hoped that for sample sizes of the order of 100,000 this would keep the normal approximations reasonably good

We conclude our discussion of Test 1 by mentioning two future lines of investigation First, the averaging formula $\bar\phi = \varphi\,(A + B_1)/2)$ is very crude We believe that weighted average such as φ of the values of $\varphi(x)$ at umber of points in the interval $(A\ \ B_1)$ will result in substantial improvement in the power of the test for all values of ϵ, that we shall be able to choose more freely concerning ourselves only with spreading the probability reasonably well over the sets S and S_1 This will make the normal approximations work much better and at the same time yield high values for the power of the test

Second, as pointed out earlier the result of the test for $H_0.\Theta = 0$ against $H\ \ \Theta = \lambda$ may well be interpreted as the result of testing $H_0\ \ \Theta < \lambda$ against $H\ \ \Theta \geq \lambda$ for the purpose of diagnosis In fact, if there is an indifference band from $\lambda - \delta$ to $\lambda + \delta$, then for the purpose of diagnosis $\Theta \leq \lambda - \delta$ may be considered to be too low for any serious concern, whereas $\Theta \geq \lambda + \delta$ may call for more careful examination of the patient For this reason, one may want to know the performance of the test statistic at various values of $\Theta \leq \lambda - \delta$ and $\Theta \geq \lambda + \delta$ This will need more elaborate study of the power of the test In this study we have studied the power only at $\Theta = \lambda$.

Test 2 The Locally Most Powerful Test

If the sample is abnormal" it contains only very small percentage of abnormal cells As pointed out earlier "normal" sample corresponds to $\Theta\ \ 0$, and an abnormal" sample corresponds to $\Theta > 0$ in the density function (1) Since we are really interested in detecting small departures from the value $\Theta = 0$ for the mixing parameter one might ant to look for the test for which the slope of the power function at $\Theta = 0$ is maximized subject to the given level of significance Such test called the locally most powerful test with the given level of significance

It can be shown that the locally most powerful test of $H_0.\Theta = 0$ against the alternative $H\ \ \Theta > 0$ at given level of significance is the one by which H_0 is accepted when

$$\sum \varphi(x_i) < C \tag{13a}$$

and rejected in favor of H when

$$\sum \varphi(x_i) \geq C \tag{13b}$$

where $\varphi(\ )$ is given in (5) and the constant C is so chosen that the test has the desired level of significance We refer to the test given in (13) by Test 2

Since the test statistic in Test 2 is agai a sum of independent random variables we attempt to find its distribution from the Central Limit Theorem However for this we need $\varphi(\ )$ to have finite variance, and that happens only when $\sigma^2 < 1\ 5$ This puts serious restriction on the pplicability of Test 2, because in most cases of interest, σ lies between 2 and 4 σ^2 lies between 4 and 16

Suppose $\sigma^2 < 1\ 5$ Then the mean of $\varphi(\ )$ is

$$m'(\Theta) = E_\Theta[\varphi(\ )]$$

$$= 1 - \Theta + \frac{\Theta}{\sqrt{2-\sigma^2}}\,e^{\sigma^2/(\ \ -\sigma^2)}$$

with $m'(\ ) = 1$ and the variance of $\varphi(\ )$ is

$$v'(\Theta) = \operatorname{var}\varphi(\ ) =$$

$$(1-\Theta)\,\frac{1}{\sqrt{2-\sigma^2}}\,e^{\sigma^2\ \ (\ \ -\sigma^2)}$$

$$+\,\Theta\,\frac{1}{\sqrt{3-2\sigma^2}}\qquad - [m(\Theta)]$$

Thus, for the value of the mixing parameter Θ

$$\bar\phi = \frac{1}{\ }\sum \varphi(x_i)$$

approximately follow Gaussian distribution with mean $m(\Theta)$ and variance $v'(\Theta)/n$ when n is large Therefore, t level of significance α, the hypothesis H_0 is accepted when

$$\frac{\sqrt{\ }\ [\bar\phi_n - m(\ )]}{\sqrt{\ '(\ )}} < \Phi^{-1}(1-\alpha)$$

and rejected in favor of H_+ when

$$\frac{\sqrt{\ }\ [\bar\phi_n - m(\ )]}{\sqrt{\ '(\ )}} \geq \Phi^{-1}(1-\alpha)$$

where $\Phi^{-1}(1-\alpha)$ is the upper $100(1-\alpha)$ per cent point of the Gaussian distribution with mean 0 and variance 1 are before The approximate power of this test for large is given as

$$\gamma_n(\Theta) \simeq 1 -$$

$$\Phi\left(\frac{\sqrt{v'(\ )}\,\Phi^{-1}(1-\ \ ) - \sqrt{\ }\ [m'(\Theta)-1]}{\sqrt{v'(\Theta)}}\right)$$

where is the sample size, nd Θ is the value of the mixing parameter at which the power is computed. Note that it is possible for $m(\Theta)$ to be less than 1. In fact, if it is less than 1 for some Θ, then it is less than 1 for all Θ. In such a case the power of the test decreases to zero as n increases. This is a most peculiar and undesirable property. For this reason we have abandoned the investigation of this test procedure.

The tables given in this paper are excerpts from a set of tables computed for a wide range of parametric combinations. A FORTRAN IV program, DATATST, written for the CDC 6400 is available from the authors. This program has been written by Mr. Henry Todd, Optical Sciences Center, University of Arizona, Tucson, Arizona.

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Transformation of Mononuclear Cells in Cerebrospinal Fluid

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The general concept of cerebrospinal fluid (CSF) cytology is based largely on investigations performed by Sayk and his co-workers. Before the introduction of Sayk's method[12] opinions on the classification of the cells were often contradictory mainly because many different preparation techniques had been used, all of which involved some degree of cell deterioration. Bannwarth[2] tried all the methods then available but found none to be satisfactory. It was even difficult to distinguish polynuclears with several methods.

The introduction of Sayk's sedimentation chamber means a more gentle handling of the cells and is thus a considerable improvement in the cytodiagnosis of CSF. Sayk's method is now widely used. After studying fixed and stained cells, Sayk classified the mononuclears in CSF into many different types and subtypes. By referring to earlier observations made by Cubie and Schmaltz and Siegmund and Schonenberg, Sayk concluded that the mononuclear cells in CSF develop from a multipotent leptomeningeal stem cell and that consequently the various mononuclear cell types represent different stages of this development.

This hypothesis can be tested by continuous observation of viable CSF cells. Muller and von Döbeln and Junker[8] used the phase contrast technique in an investigation of viable CSF cells but did not observe any transformation of one cell form into another, probably because the cells were damaged or destroyed by the centrifugation.[1,8]

The main purpose of the present investigation was to study the ability of mononuclear cells in CSF to undergo transformation from one cell form into another. Viable cells, after gentle sedimentation, were studied first in phase contrast and then after fixation and staining.

METHODS AND MATERIAL

The method has been described in detail earlier. Under the phase contrast microscope the main cell types of CSF can be identified. The number of mononuclears, polynuclears and erythrocytes was obtained by use of a Jessen chamber (10 cmm) and phase contrast microscope. When the chamber contained >100 white cells, a differential count was performed as well. In order to obtain a differential count on samples containing <100 white cells per 10 cmm or to produce stained preparations, a simple sedimentation chamber was used. One end of a glass cylinder of 4 cm length and 1 cm inner diameter is dipped in melted vaseline and immediately thereafter applied to a clean, fat-free slide. As the vaseline cools and solidifies, it fixes the cylinder to the slide. About 0.7 ml of fresh CSF was then deposited in the cylindrical chamber. After one hour the supernatant is sucked off with a Pasteur pipette. About 0.2 ml of the fluid is left and the cylinder is removed from the slide, leaving behind a ring of vaseline enclosing the sedimented cells. The preparation is examined in a phase contrast microscope with a water immersion lens (x40, ocular 12.5 wide angle). When the sample is poor in white cells (<100 per 10 cmm), a differential count is performed at this stage of the examination. One hundred cells were counted. The residual fluid is withdrawn and the preparation air-dried and stained with May-Grünwald-Giemsa.

On samples that are bloody, examination in phase contrast is not possible. The total cell count is done with conventional methods. To obtain a stained slide about 1 ml of the sample is allowed to sediment for one hour in the sedimentation chamber. The supernatant is withdrawn with a Pasteur pipette, the cylinder removed from the slide and the sediment washed with a strong jet from the pipette. A large number of the erythrocytes is thereby washed away and a monolayer of white and red cells is left on the slide. The preparation is then air-dried and stained with May-Grunwald-Giemsa.

When the sample contains many leukocytes, as for instance in meningitis, sedimentation for one hour is too long. The result is a thick deposit in which it is impossible to study individual cells under the microscope. In these cases sedimentation is limited to a few minutes.

Indian ink was used for studying the phagocytizing capacity of the cells. At the beginning of the sedimentation a drop of Indian ink was added to the sample. After one hour the preparation was stained with May-Grünwald-Giemsa.

*the mixing parameter at which the power is com*puted. Note that it is possible for m (Θ) to be less than 1. In fact, if it is less than 1 for some Θ, then it is less than 1 for all Θ. In such a case the power of the test decreases to zero as n increases. This is a most peculiar and undesirable property. For this reason we have abandoned the investigation of this test procedure.

The tables given in this paper are excerpts from a set of tables computed for a wide range of parametric combinations. A FORTRAN IV program, DATATST, written for the CDC 6400 is available from the authors. This program has been written by Mr. Henry Todd, Optical Sciences Center, University of Arizona, Tucson, Arizona.

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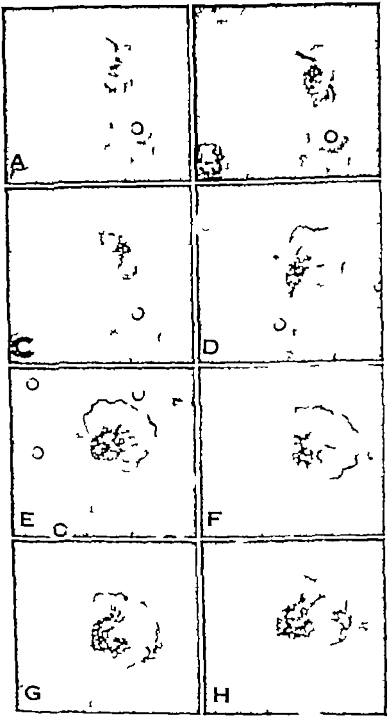

Fig. 1 Transformation of single monocytoid into macrophage ( ) The monocytoids settles on the glass slide (b) After 30 seconds (c) After 1 minute (d) After 1 minute 10 seconds ( ) After 2 minutes (f) After 3 minutes (g) After 4 minutes (h) After 5 minutes Phase contrast (X 400)

All examinations were done at room temperature

The material consisted of CSF cells obtained from healthy subjects and from patients suffering from various neurologic diseases, such as hemorrhagic and nonhemorrhagic stroke multiple sclerosis, polyneuritis, intra- and extracerebral tumors, and acute meningoencephalitis. Sediments from about 3000 patients were studied.

In the following "cellular activity" and "transformation activity" are to be understood only as the morphologic changes observed in the cells, such as formation of folds or vacuoles in the cytoplasm, the development of pseudopodia, and transitory changes of cellular density

RESULTS

Examination in Phase Contrast Microscope

In the samples examined after a few minutes of sedimentation the freely suspended cells could be studied before they reached the glass slide This was made possible by keeping a cell constantly in focus on its way down to the bottom.

Lymphocytes attached poorly to the slide Most of them retained their spherical form unchanged. A few lymphocytes showed a small cytoplasmic "tail" that sometimes increased or decreased in association with the development or disappearance of folds. Occasional lymphocytes took on a "hand-mirror" appearance (cf Fig 2) The nucleus did not change during this activity of the cytoplasm.

Freely suspended in the fluid, the monocytoids were more or less *spherical before they reached* the slide, but from many of them thin or broad pseudopodia were seen to project or withdraw The monocytoids varied in size as well as in density Some were only slightly larger than lymphocytes, differing from them by a higher density and by a tendency to send out marked pseudopodia. Others were large sometimes 30 to 40 μ in diameter The larger the cells, the denser they *tended to be The nucleus could not* be distinguished from the cytoplasm, but vacuoles were discernible.

When the monocytoids reached the slide they attached within seconds. Some of them underwent rapid morphologic changes (Fig 1) The pseudopodia observed during sedimentation spread out on the glass, elongated, and gradually established contact with other broad cytoplasmic pseudopodia, *which had in the meantime extended* from other parts of the cell. At the same time the outline *of* the nucleus began to be discernible the nuclear membrane emerged *and became* gradually more distinct, while the nuclear structure became looser Within a few minutes, *the original* cell had transformed into a typical macrophage with a voluminous cytoplasm and *a round oval* or kidney-shaped nucleus.

Many of the monocytoids without pseudopodia were converted to macrophages in another way *The sharply* demarcated cell body was suddenly surrounded by a faintly discernible external circular *cytoplasm. At the same time as the* external part of the cytoplasm gradually became denser the nucleus decreased in density became elongated, and finally assumed the appearance of the nucleus of a macrophage. The "new-formed" cytoplasm had in the meantime become irregular in outline and the cell appeared as a typical macrophage.

Other monocytoids seemed to begin a trans*formation to macrophages, but after a while the* cytoplasmic processes were withdrawn and the cells returned to their *original appearance.*

Some monocytoids, preferably the smaller ones, developed only small pseudopodia slowly extending round the cell, which at the same time flattened out as the nucleus became looser and more readily discernible The density of the nucleus and of the cytoplasm could vary from one minute to the next. Sometimes they were of equal density with the result that the nuclear *structure could not* be *distinguished from the* cytoplasm. The cells assumed varying shapes and forms and changed from one form to another Most of them were irregular in outline and therefore appeared "angular" On continuous observation such a cell was sometimes seen to transform into a macrophage in the course of 20 to 30 minutes. *Thread-like tentacular processes extended* from the cytoplasm, gradually increasing in volume and density and spreading round the cell which finally assumed the characteristic appearance of a macrophage.

Many monocytoids, however attached to the glass without undergoing any changes during the time of observation, which amounted to one-half to 1 hour

In samples examined after 1 hour *of sedimentation*, monocytoids were seen as round forms and as "angular" forms Occasional round monocytoids were still seen transforming into macrophages. On the whole the number of macrophages was constant during a subsequent observation period of one half to 1 hour Many "angular" monocytoids underwent morphologic changes, but at a slower rate than those cells examined *after a few minutes of sedimentation*

During continued observation after the completion of the transformation, many macrophages showed a striking activity Their cytoplasm was undulating sharp folds appeared like ridges in the cytoplasm, and pseudopodia were extended *in different directions. The pseudopodia could fuse together into large masses of cytoplasm* and in this way the cell changed shape completely within minutes The formation and disappearance of vacuoles could easily be followed.

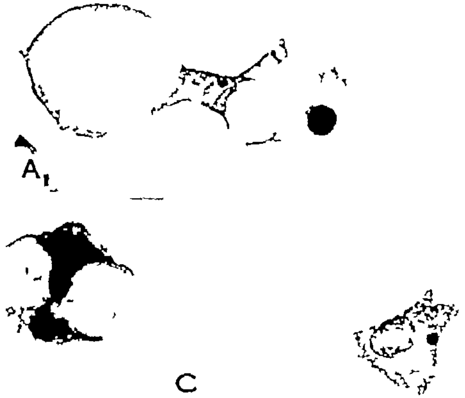

Fig 4. Bizarre types of monocytoids (a) In virus encephalitis (b) In myelitis of unknown cause (c) In subarachnoid haemorrhage of 7 days duration May-Grunwald Giemsa (× 987)

condition to another It was lowest in samples from healthy persons In these samples, small, distinct, sparse granules were seen in only 10 to 30 per cent of the monocytoids and macrophages The number of macrophages was also small

In the CSF where the cellular activity was lively most, sometimes all, monocytoids and macrophages showed signs of phagocytosis Granules were large and numerous (Fig 5) Some cells contained conglomerates of Indian ink This type of CSF contained numerous macrophages Phagocytosis was most pronounced in cases of subarachnoidal hemorrhage and hemorrhagic infarction Maximum phagocytosis was observed some days after the onset of the diseases, when 80 to 100 per cent of the cells were loaded with granules In subarachnoidal hemorrhage this occurred at the same time as the appearance of erythrophages

DISCUSSION

Sayk introduced nomenclature and classification of the monocytoids that are now generally accepted He believed these cells to be reticulo-histiocytic cells and classified them according to morphologic criteria into three fundamental forms lymphocytic, monocytic and plasmocytic Each of the fundamental forms had basic form, transitional form, stimulated form, and a special form ("Sonderform) Reticulocytoid cells belonged to special forms, monocytoids resembling blood monocytes and fibrocytic cells resembling fibroblasts, and macrophages As macrophages he classifies all mononuclear cells that have phagocytosed visible substances (hemosiderin, hematoidin, erythrocytes and other cells, fat, etc)

Sayk assumed that all the described monocytoids develop from multipotent leptomeningeal stem cell

Ever since the end of the nineteenth century it has been debated whether lymphocytes can differentiate into other types of cells So far it has not been shown that they can differentiate into cells of the granulocytic or erythrocytic series On the other hand lymphocytes can, under certain conditions transform into macrophages This was observed by Rebuck *et al.* who studied

In neurologically healthy persons cellular activity was low in CSF The macrophages, which were few in number showed insignificant changes in the cytoplasm. Slight wrinkling occurred but only rarely vacuoles whereas a certain perinuclear granulation was common The monocytoids were characteristically inactive and they varied little in appearance

In some diseases, particularly in acute meningo-encephalitis, subarachnoidal hemorrhage, intracerebral hematoma, and embolic cerebral infarction, as well as after pneumoencephalography the monocytoids and the macrophages showed a wide variety of morphologic changes and the monocytoids showed a striking transformation activity In such CSF the most bizarre cell forms could be seen

Fig 2 Two lymphocytes with hand-mirror appearance May-Grünwald-Giemsa (× 1000)

Examination of Preparations Stained with May-Grünwald-Giemsa

The fixed and stained cells reflected the cellular activity observed in phase contrast microscopy The cytoplasm of the macrophages was only lightly basophilic, but it was readily recognized. The shape and appearance of the nucleus and cytoplasm of the macrophages and the monocytoids were the same as under the phase contrast microscope as were also the pseudopodia of the monocytoids and the lymphocytes with a "hand-mirror" appearance (Fig 2)

Of special interest were the slides prepared from CSF containing different forms of macrophages and monocytoids which at the phase contrast examination were characterized by vigorous transformation activity These preparations contained a number of monocytoids among which it was easy to trace transitions from one cell type to another (Fig 3) The longer a monocytoid had progressed on its transformation to a macrophage the less basophilic was its cytoplasm Concomitantly the chromatin net of the nucleus tended to become looser This type of CSF showed a great variation of the morphologic appearance of the monocytoids (Fig 4) Some of them could easily be mistaken for tumor cells

Study of the Ability of CSF Cells to Phagocytize Indian Ink

Only monocytoids and macrophages proved able to phagocytize Indian ink The lymphocytes never contained any phagocytosed granules

The degree of phagocytosis varied from one

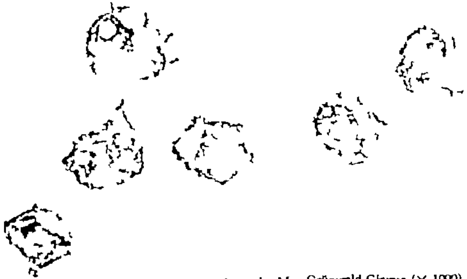

Fig 3 Monocytoids after air-encephalography May-Grünwald-Giemsa (× 1000)

migration of mononuclears to the site of inflammation Peripheral lymphocytopenia and monocytopenia did not affect the migration of mononuclears He could not demonstrate gradual transition from lymphocytes to monocytes at the site of inflammation, as described by R bock. Liblinsky concluded that the mononuclears reached the site of inflammation mainly from the tissue mesenchyme and not from the blood.

It is therefore possible that polymorphonuclear cell reaction in the CNS is *one factor* that stimulates its RES, resulting in an outflow of activated monocytic cells into the CSF

According to Junker[8] Sayk[14] and others, the CSF contains fibroblasts or fibrocytes These cells, however are perhaps macrophages with long, cytoplasmic processes or monocytoids that has assumed fibrocytic appearance after having attached to the glass During the continuous observation of viable monocytoids and macrophages, individual cells can be seen undergoing transformation to assume forms and appearances which closely resemble those of fibroblasts

Ivers and Pomerat, who cultured human cancer cells from ascites, emphasized the difficulty in distinguishing normal cells from tumor cells because of the various shapes and forms of the macrophages Some of the macrophages presented very bizarre appearance These writers considered the many different forms to be manifestation of different stages of transformation This also applies to CSF It is important for the examiner to be closely acquainted with the shifting appearance of monocytic cells in CSF otherwise they may readily be mistaken for tumor cells

Sayk and his school classify as macrophages all mononuclear cells that have phagocytosed visible substances (hemosiderin, erythrocytes and other cells, fat, etc) It would seem more appropriate to apply the term macrophage to those monocytoids that are transformed on glass to large cells rich in cytoplasm, as described above (Fig 1). For all mononuclear cells, thus also macrophages, which have phagocytosed visible substances, the term mononuclear phagocytes should be used

Thus, it is important from clinical point of view to distinguish macrophages as particular group Most diseases of the CNS that cause cytologic changes in CSF are accompanied by more or less pronounced reaction of the macrophages A relative increase in the number of macrophages in the CSF with normal total cell count is sometimes the only sign of a pathologic process in CNS

Since the monocytoids in the CSF have strong tendency to undergo transformation on glass, it can readily be appreciated why it has been difficult to classify them into a rigid static system with types and subtypes *ad modum* Sayk. It is also doubtful whether such a classification would serve any clinical purpose

SUMMARY

Viable human CSF cells sedimented on glass were studied under the phase contrast microscope and after staining with May-Grünwald-Giemsa.

1 The CSF invariably contained lymphocytes and spherical monocytoids When the monocytoids attached to the glass, many of them changed in form and appearance During the transformation process, some monocytoids developed into macrophages Lymphocytes underwent no transformation

2. All different forms of monocytoids, including macrophages, could phagocytose Indian ink *in vitro* and erythrocytes and hemosiderin *in vivo* It was therefore concluded that these cells belong to the reticuloendothelial system

3 The degree of transformation was correlated to the degree of phagocytosis and number of macrophages It was low in normal CSF but high in certain diseases of CNS

4 In conditions which caused a polymorphonuclear leukocyte reaction in the CSF the monocytoids and macrophages underwent striking morphologic changes and lively transformation. There is thus reason to believe that polynuclear cell reaction in the CNS stimulates an outflow of activated" monocytoids the CSF

5 Classification of monocytoids on a morphologic basis int different types and subtypes was almost impossible because of the strong tendency of these cells to undergo transformation

ACKNOWLEDGMENTS

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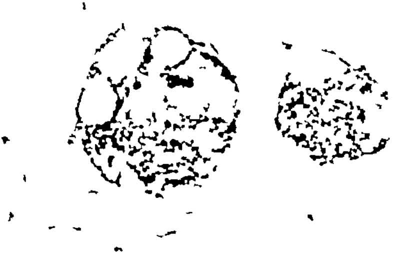

FIG. 5. Two erythrophagocytes that have phagocytosed Indian ink in vitro. Subarachnoid haemorrhage 3rd day May-Grünwald-Giemsa (× 1000)

cells from cantharides blisters. On several occasions, they observed the *living individual* lymphocytes as they progressively transformed into small histiocytes or macrophages, increasing cytoplasmic size and ingesting foreign material into them a process that took about 30 minutes

Jacoby[4] expressed the opinion that the transformation problem is not solved and that direct proof is lacking

In the present investigation, no CSF lymphocyte *was seen to transform to a macrophage in the* way described by Rebuck *et al* The CSF "round cells" that possessed this property were monocytoids.

The present investigation also showed that the monocytoid and macrophages phagocytize India ink *in vitro* and erythrocytes as well as hemosiderin *in vivo* According to the criteria set up by Aschoff[1] for the reticuloendothelial system (RES) *these cells belong to this system*

There is no reason to believe that the transformation of the monocytoids to macrophages, described here, was associated with any new formation of cytoplasm or other cellular material especially since the transformation required only a few minutes. The morphologic changes which *monocytoids* in CSF underwent on glass occurred in essentially the same way as described in cultures of mammalian exudate cells. *According to Jacoby*[4] monolayer cultures of such cells begin mainly as small, round or oval *monocytoids* Many of them give off pseudopodia Then the cell picture gradually changes. Some cells increase *in size*, and various, often bizarre forms develop. Others retain their rounded form and size One individual cell can be seen to transform into any other type cell occurring in the culture All the different cells, *irrespective of appearance* are capable of phagocytosis.

The monocytoids in the CSF are spherical. On contact with glass they attach to it, and it seems that this attachment serves as one factor necessary for its initiation of a transformation process. Some cells develop into large macrophages whereas others persist in the monocytic stage in which they can undergo a variety of changes in shape. Some cells do not change.

However *attachment to glass is not enough per se* to initiate the transformation of the monocytoids, *otherwise* most of them *would develop into* macrophages, which is obviously not the case. The cells that are transformed to various forms must have been stimulated already *in vivo* resulting in a characteristic change in morphology after contact with the glass slide in the bottom of the sedimentation chamber Some monocytoids thus appear to be predestined to develop into typical macrophages.

The strongest macrophage monocytoid reaction was observed in samples from patients in whom *the CSF* showed a pronounced polynuclear reaction, as in acute meningo-encephalitis, subarachnoidal hemorrhage intracerebral hematoma, and hemorrhagic cerebral infarction [illegible]

According to Opie, "it is not impossible that polynuclear leukocytes together with other products of tissue degeneration, serve as the principal stimulus to the activity of mononuclear cells Page and Good demonstrated an association between failure of lymphocytic migration and transformation to macrophages in experimentally *induced neutropenia in rabbit Using a modified* Rebuck skin window technique Libánsky studied the source of mononuclears *at the site of inflammation* in patients with hemoblastosis. Some of them had peripheral neutropenia or lymphopenia or monocytopenia. Libánsky found that peripheral neutropenia was regularly followed by decreased

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A Classification of Nuclear Aberration in Relation to Malignancy Associated Changes (MAC)

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In 1959 Nieburgs, and others, reported their observation of systemic morphologic change in nuclei of mature cells from benign appearing tissues distant from malignant tumors in the human and during developement of tumors in animals.

In 1962, the same laboratory published results of research for this change in normal appearing buccal cells of cancer patients. They reached a diagnostic accuracy of 72 per cent in discriminating between the patients with distant cancer and non cancer surgical patient controls.

More recent work by this group has related variations of nuclear malignancy associated changes (MAC) to the mitotic phases.

Others[illegible] have attempted to reproduce these findings with variable success. In our experience diagnostic accuracy was low but statistical evaluation of the diagnostic distribution attained significance. This implied that the variable was tested, the criteria, existed. However the criteria for malignancy associated changes are not quantitative, and may be subject to misinterpretation. The number of cells with MAC is thought to vary between different cases, and at varying times within one case.

The purpose of this study was to attempt to demonstrate the spectrum of Type A, telephase related, morphologic changes that may be seen, and the degree to which they are present, and the amount of variation over a period of time.

METHODS AND MATERIALS

Buccal smears were taken by firm tongue blade scrapings on 24 patients for this study. The normal controls consisted of fourteen dental students. These subjects had no evidence of current disease or past history of severe disease. For disease controls (patients with psoriasis) sequential smears were taken on a weekly basis. None of these patients had a past history of cancer or any other severe disease in the past or present except psoriasis. Patients with psoriasis were selected because of the use of Methotrexate in treatment for this disorder (Cases 2, 3 and 4) and because the disease itself implies an epithelial proliferation disturbance.

The experimental cases consisted of six cancer patients with varying stages of disease at sites distant from the mouth. Table I shows the histologically confirmed diagnosis of each cancer patient. These patients were smeared sequentially on a morning, afternoon, and evening basis when possible so that some observation could be made as to whether the occurence of MAC or MAC like morphology was related to circadian rhythm. The smears were taken in each quadrant of the buccal mucosa of all patients. The slides were labeled, fixed, and stained by the Papanicolaou method. Each case situation was represented by two slides as a methodology control. The entire series of 90 slides were randomized and read without knowledge of individual slide origin.

It was arbitrarily decided that only 100 cells would be counted and classified on each slide. Since no actual quantitative definition of the MAC-negative buccal smear has been made it was hoped that this relatively low number would help in future definition of this state.

A Zeiss RA 38 Microscope with planapochromatic objectives was used for this study. In examining each slide areas were sought that were representative with the scanning objective (25×). By this means areas representative of the entire slide were selected. Coherent clumps were included in the counted area to the degree that they were present.

Once an area was selected with scanning power the 40× high-dry objective was used to examine, count, and classify the nuclei in the field. This was done without moving the slide until every cell in that high-dry field has been classified. A standard percentage blood cell calculator was used for recording the findings.

It must be noted that the optimum magnification for confirming the MAC is 1000× oil immersion, and that the results from this study relate only to those abnormalities seen at 400×.

The cells were classified according to the following criteria: "0"—superficial cell nucleus, pyknotic (Fig 1). "1"—prepyknotic nuclei, obviously normal, with an ovoid nucleus and normal N/C ratio (Fig 1). "2"—normally maturing nuclei, round to

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This paper was presented in part at the 48th Annual Meeting of the International Association for Dental Research.

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Buccal smears were taken by firm tongue blade scraping on 24 patients for this study. The normal controls consisted of fourteen dental students. These subjects had no evidence of current disease or past history of severe disease. For disease controls (patients with psoriasis) sequential smears were taken on a weekly basis. None of these patients had a past history of cancer or any other severe disease in the past or present except psoriasis. Patients with psoriasis were selected because of the use of Methotrexate in treatment for that disorder (Cases 2, 3, and 4) and because the disease itself implies an epithelial proliferation disturbance.

The experimental cases consisted of six cancer patients with varying stages of disease at sites distant from the mouth. Table I shows the histologically confirmed diagnoses of each cancer patient. These patients were smeared sequentially on a morning, afternoon, and evening basis when possible so that some observation could be made as to whether the occurence of MAC or MAC-like morphology was related to circadian rhythm. The smears were taken in each quadrant of the buccal mucosa of all patients. The slides were labeled, fixed, and stained by the Papanicolaou method. Each case situation was represented by two slides as a methodology control. The entire series of 90 slides were randomized and read without knowledge of individual slide origin.

It was arbitrarily decided that only 100 cells would be counted and classified on each slide. Since no actual quantitative definition of the MAC-negative buccal smear has been made, it was hoped that this relatively low number would help in future definition of that state.

A Zeiss RA 38 Microscope with planapochromatic objectives was used for this study. In examining each slide, areas were sought that were representative with the scanning objective (25×). By this means areas representative of the entire slide were selected. Coherent clumps were included in the counted areas to the degree that they were present.

Once an area was selected with the scanning power the 40× high-dry objective was used to examine, count, and classify the nuclei in the field. This was done without moving the slide until every cell in that high-dry field has been classified. A standard percentage blood cell calculator was used for recording the findings.

It must be noted that the optimum magnification for confirming the MAC is 1000× oil immersion, and that the results from this study relate only to those abnormalities seen at 400×.

The cells were classified according to the following criteria: "0"—superficial cell nucleus, pyknotic (Fig. 1). "1"—prepyknotic nuclei, obviously normal with an ovoid nucleus and normal N/C ratio (Fig. 1). "2"—normally maturing nuclei, round to

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This paper was presented in part at the 48th Annual Meeting of the International Association for Dental Research.

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be noted that, at one time or another four of the patients bearing cancer were identified with that disease by that finding

It has been stated that the complete MAC nucleus (Type 5) is related to cancer while incomplete forms (Type 4) are not. Statistical analysis (Fisher' exact probability) of the diagnoses during the entire series resulted in statistical significance of the 5-type cell finding ($p = 0014$) (Table V) This significance reflects the review of several sequential smears, 100 cells examined each time, and indicates that criteria for the nucleus of complete MAC influenced the cancer noncancer differentiation under those conditions

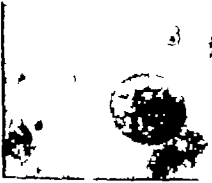

Fig. 2. Type 3 cells (maturation impairment) Compare size increase and circular tendency over normal cells of Fig 1 (400×)

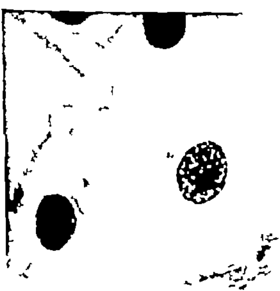

Fig. 3. Type 4 cell (incomplete MAC) Note size increase over normal (Fig 1) and density of chromatin subsequent nuclear spaces unequal in size and staining quality

ovoid with fine reticular chromatin patterns (Fig 1) 3°—marked N C ratio change, marked increase in the nuclear size and lightly stained heterochromatin in a *round, not ovoid nucleus.* (This cell reflects the similar manifestation to those seen in association with pernicious anemia.[7]) (Fig 2) 4°—similar cell to the preceding type with increased heteropyknocity of the chromatin, and irregular and imperfect nuclear clear area (Fig 3) 5°—nuclei representing complete MAC, formerly described[3] (Fig 4)

Although this classification is arbitrary it is hoped that it would record part of the spectrum of changes seen in various nuclear aberrations.

RESULTS

The normal cell types (0 1 and 2) varied without any apparent pattern and were collectively predominant. Therefore for tabulation of the results, only the 3 (maturation impairment) 4 (maturation impairment plus heteropyknocity) and 5 (complete MAC) type cells are included.

A *Methodology Control*

On paired slides, a *reasonable similarity* in count of 3, 4 and 5 cells was recorded in all but eight of the 45 case situations (Table II) Reasonable similarity is defined here as the total of abnormal cells recorded with less than a 10 cell variation between slides. Seven of the wide variations occurred in patients with cancer and one occurred in normal controls.

B *Experimental Results*

Table III shows the findings of abnormal cells in the control series. Note that most cases were seen to have type three nuclei to some degree, but that in no instance were type 5 nuclei seen in this group. The patients with psoriasis had an apparently in*significant increase in the type 3 category*

More abnormal nuclei were counted in *the* cancer series than either of the control groups (Table IV) The number of abnormal cells are small under these conditions and only 3 of the cancer cases had a significant number of 5 and 5-type nuclei. All but one cancer case had the incomplete type of MAC (4) at one time or another. Six control cases also fall in the category of having 4-type cells, but the percentage (24%) is far below that of the cancer series.

If the 5-type cell represents classic MAC, it may

TABLE I

Case	Age	Sex	Disease
C-1	69	F	Hepatoma
C-2	69	M	Ca Pancreas
C-3	64	M	Metastatic melanoma
C-4	59	F	Carcinomatosis and dehydration
C-5	55	M	Ca colon with liver metastasis
C-6	31	M	Ca thyroid no metastasis demonstrated

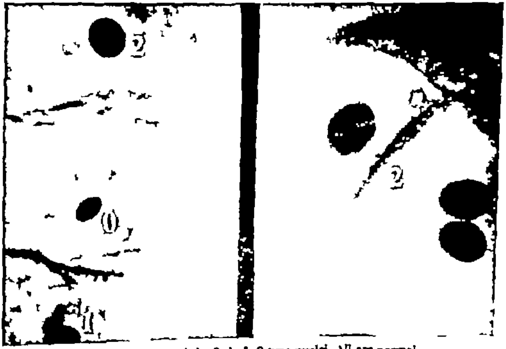

FIG. 1 As indicated the 0, 1 & 2 type nuclei. All are normal. Note size variation with similar magnification (400×)

TABLE IV

Experimental Case (Cancer)

Case	Day 1, AM			Day 1, PM			Day 2, AM			Day 2, PM			Day [illegible]		
	Cell Type														
				3			3			3					
C-1	9	—	—	7	—	—									
	3	—	—	12	—	—									
C-2	3	10	—	11	14	7									
	1	5	5	8	17	4									
C-3	11	—	—	10	3	—	11		—	3	—		3	1	—
	2	31	6	6	18	7	13	5	2	14	—		15	2	
C-4	8	3	—	14	16	1							27	4	1
	18	2	—	—	—	—							13	1	
C-5	3	—	—	6	2	—	2	—	—						
	—	—	—	11	6	1	6	2	—						
C-6				—	—	—	1	—	—	2	—	—			—
				2	—	—	6	3	—	—	—		2		—

TABLE V

Diagnosis of MAC by Complete MAC Nuclear Morphology

	+	−
Cancer	4	2
Controls	0	18
	p = .0014	

TABLE VI

Diagnosis of MAC by Incomplete MAC Nuclear Morphology

	+	−
Cancer	5	1
Control	6	12
	p = .048	

an increased number were seen in the experimental cancer cases.

DISCUSSION

There was variation greater than 10 per cent in total amount of type 3, 4 and 5 cells in eight duplicate-slide situations of the forty-five pairs (Table II). This may reflect one or two possibilities. In one respect, this may represent inaccurate control and or imperfect interpretation of the cells on the slide. A second factor may have influenced the paired slide methodology control. This factor may be local inflammatory disease and or minor traumatic disorders related to opposite sides of buccal mucosa.

No conclusions can be drawn regarding diurnal or circadian rhythm effect on the finding of abnormal cell nuclei. However wide variation was noted between specimen collection times in Cases C3, C4, and C5 (Table IV). The cases of hepatoma and thyroid carcinoma (C1 and 6) were consistently negative since no observation of complete MAC were recorded. Only once in the six slide pairs involving these two cases were suspicious cells (4-type) seen. The case with carcinoma of the colon (C5) had minimal findings of MAC and MAC-like cells although well documented liver metastasis was present.

The most obvious MAC positive cases consist of the remaining experimental cases: one with widespread melanoma (C3) was found positive in three of five slide-pairs, the other with carcinomatosis (C4) was positive in two of three slide-pairs, and the case of carcinoma of the pancreas (C2) was positive on both of the survey occasions.

These data suggest that a variable increase in the number of nuclear abnormalities can be seen in cancer series when compared to controls. The changes observed are related to the MAC concept described by Nieburgs, although the findings were recorded while using less than optimum magnification suggested for confirmation of that circumstance.

CONCLUSION

1. An increased number of abnormal nuclei according to our classification occurred in the cancer series than in the controls.

2. Variation in amount of MAC-type morphology over a period of two days has been recorded in our cancer series.

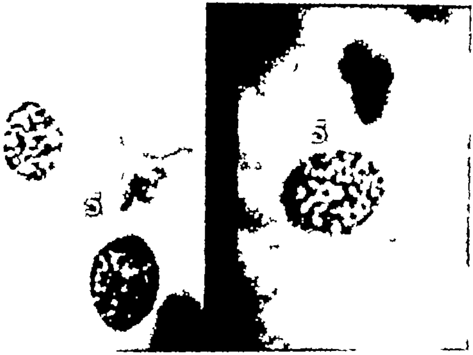

Fig 4 Type 5 cell (complete MNC) Note orderly pattern, pyknosity of chromatin perfectly clear nuclear spaces Note that this cell varies only in degree from Type 4 cell.

TABLE II

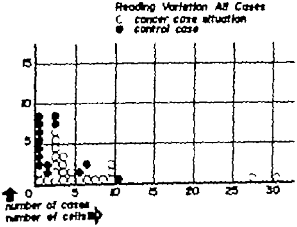

TABLE III

Controls							
Prisoners				Normal			
	C D Type				Cell Type		
Case		4		Case			
P 1 A	3	—		N 14			—
	12						
P 1 B	15	—		N 18			
	14	3					
P 1 C				N 21	1		
					2		
P 2 A				N 45			
	1	2			3		
P 2 B	4			N 57			
	5	—	—		—		
P 2 C	1	—		N 61			
					—	1	
P 3 A	7			N 78	8		
	5	—					
P 3 B	6			N 85	4		
		—					
P 3 C	2			N 84			
			—			—	
P A			—	N 107			
		—	—				
P 4 B				N 111		—	
	1						
P 4				N 1[illegible]	—		
	—					—	
				N 158			
					1	1	
				N 133	1	—	
					1	—	

By comparison, application of this statistical evaluation to the type 4 cell finding resulted in significance (p = .018) but less so than the 5 type cell (Table VI)

In other words, criteria for both of these types of nuclei (4 and 5) significantly affected the 2 × 2 Table distribution, but to a decreased degree in the 4-type cell. The lack of sequential smears in the control series precludes our interpretation of this data as any other than a comparison of the 4 and 5 type nuclei

The 5-type cell was seen in all but 6 cases, but

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DNA Values from Cytology in a Case of Palatal Carcinoma

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Case Report

A seventy-two year old man (J. M.) was hospitalized with a tumor on the right side of the soft palate. The tumor had been noted five to six months before admission and had been slowly increasing in size since. When first seen at the hospital it was ulcerative (Fig. 1) and the submandibular lymph nodes were palpable at the right side. An exploratory excision from the tumor was performed and histologic examination revealed a polymorphous carcinoma with many mitoses (Fig. 2). Smears of the tumor surface for characterizing the tumor by DNA-measurements were made at the same time and showed small to medium sized pale nuclei with enlarged nucleoli and narrow margins of cytoplasm. Remarkable are multipolar, bipolar and unipolar mitoses (Figs 3 to 5). The poor condition of the patient, suffering also from diabetes mellitus, as well as his lack of cooperation impeded the operation. He was sent by his family to a non-medical practitioner where he was treated with at least eight different homeopathic preparations. One of them contained extracts of Viscum album the effects of which on tumor growth has been studied by various authors[1-4]. While tissue cultures gave no significant results, in an experiment with mice an ascitic tumor lived longer after pretreatment with extracts of Viscum album. During the following months the ulceration healed and the tumor became smaller. When we saw the patient five months later he had gained weight and felt better but still refused surgical treatment. At this time smears from the tumor surface contained only epithelial cells. Nine months after continuous treatment with the homeopathic drugs hospitalization became necessary again. The patient's condition had considerably deteriorated. The tumor had grown and x-ray

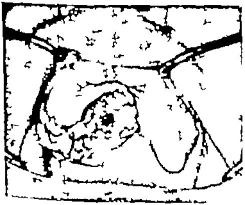
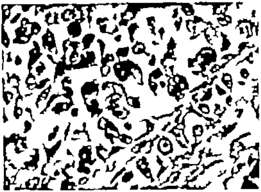


Fig. 1 Ulcerating tumor of the soft palate which had developed about 6 months.



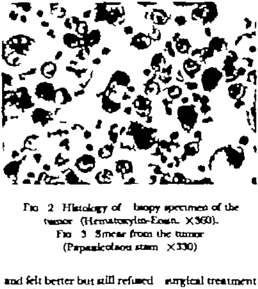


Fig. 2 Histology of biopsy specimen of the tumor (Hematoxylin-Eosin ×360).
Fig. 3 Smear from the tumor (Papanicolaou stain ×330).

3 There was an increased significance of the 5-type nucleus (complete MAC) over the 4-type (incomplete MAC) in the 2 × 2 diagnostic tables The attained significance in no way indicates diagnostic accuracy for cancer in this study because the control series was not sequentially smeared, and only one type of abnormality other than cancer was included in this series.

4 Methotrexate treatment of psoriasis did not appear to influence the degree of abnormal nuclei seen.

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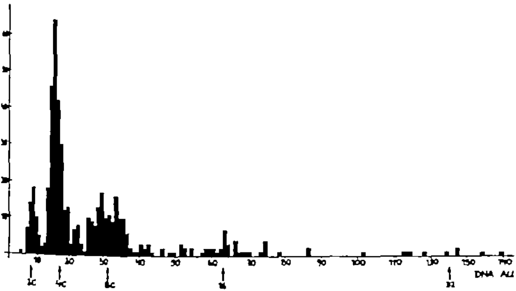

FIG. 6. Histogram of DNA content of 500 tumor cell nuclei in smears

quite high … measured in the case of the so-called alveolar cell carcinoma but … can be expected in … case where smears are made from the tumor itself

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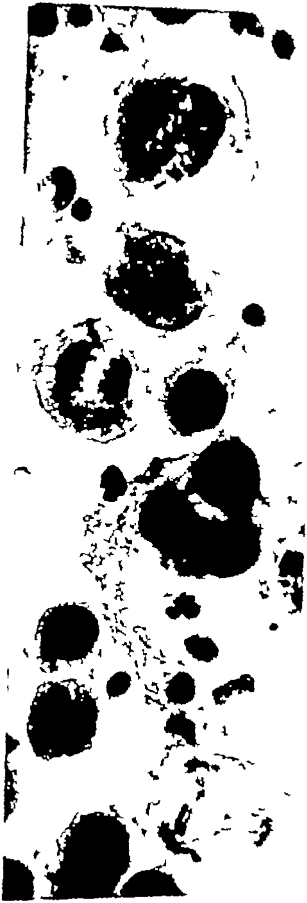

FIG. 4 Multipolar mitoses in smear (Papanicolaou stain. ×1,000)

FIG. 5. Unipolar mitosis in a smear (Papanicolaou stain. ×1 000)

examination demonstrated local bone destruction. One month after the second admission the patient died of heart failure. Autopsy revealed local tumor growth but no metastases were found. In addition hepatic cirrhosis and a pronounced arteriosclerosis were observed

MATERIALS AND METHODS

We measured the total DNA content of 500 Feulgen-stained nuclei with the scanning cytophotometer UMSP I/Zeiss with digital fast scanning attachment ND 50[3] The diploid value in arbitrary units was determined by measuring lymphocytes

RESULTS

The histogram (Fig 6) showed peaks at C, 4 C, 8 C, and 16 C as well as some nuclei with 32 C. However there were many intermediate values and no clear cut limits exist between the classes Therefore polyploidization can be demonstrated by the distribution of the peaks but the intermediate DNA-values may be explained by aneuploidy resulting from the atypical mitoses or by DNA synthesis[4-7]

DISCUSSION

In the published case of a so-called alveolar cell carcinoma only the classes 3 C and 4 C as well as 6 C to 8 C formed block and the other classes were separated while multipolar mitoses were seen as frequently in the histologic preparations as in this case Therefore we feel that the variable DNA values in this case are due to DNA synthesis It is unlikely that DNA synthesis continued in the nuclei of the exfoliated cells of

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We thank Prof. Dr L. E. Feinendegen, Director of the Institute of Medicine at the Kernforschungsanlage Jülich, for the permission to perform the measurements in his laboratories.

ing to licensure and approval of cytology laboratories of interstate commerce

Dr Hicklin.

The current Federal requirements relating to the practice of cytopathology are contained in three documents:

1 The Clinical Laboratories Improvement Act of 1967 (CLIA)

2 The Code of Federal Regulations Title 42, Chapter I, part 74 Regulations for Licensure of Clinical Laboratories.

3 The Code of Federal Regulations Title 20, Chapter III, Part 405 "Conditions for Coverage of Services of Independent Laboratories, relating to the Health Insurance for the Aged program of the Social Security *Administration*

In these various sources the terms cytology and exfoliative cytology are interpreted to mean the examination of scraped, washed, or exfoliated cells, body fluids, or exudates for (a) detection of cancer, possibly pre-cancerous or benign lesions, infections or infestations, (b) monitoring treatment, (c) screening of normal from non-normal, and (d) the assessment of hormonal activity in the female For purposes of current licensure cytology does not include cytogenetics, or tissue culture methods for diagnosis of connective tissue or metabolic diseases. Many prefer "cytopathology" which will be used synonymously with cytology and exfoliative cytology

Most of the relevant details are contained in the Public Health Service Regulations. However it is perhaps in order first to summarize briefly the Clinical Laboratories Improvement Act of 1967 enacted by the 90th Congress Section 5 of Public Law 90-174 amends Part F of Title III of the Public Health Service Act to include clinical laboratories under the licensing authority which previously had extended only to biological products. The amendment defines clinical laboratory interstate commerce and general scope of licensing procedure with provisions for revocation and redress, exceptions and exemptions, violations and the setting of penalties "Cytological examination is included specifically

Essential to the development of our subject is Sec 353 (d) (1) which, in summary specifies that whatever standards *for cytology laboratory* operation are devised by the Secretary or his agency must include ( ) a *quality control* program, and (b) participation in a *proficiency testing program* established by the Department of Health, Education and Welfare, in addition to (c) the professional requirements of the personnel and (d) the physical requirements of the laboratory operation

Federal Regulations are the creation of the executive branch, under the broad limits set down by the legislative. Federal Regulations are not necessarily formulated by majority vote of a deliberative body but wide-ranging expert advice is usually sought in their drafting and diverging views and arguments are invited before they are finalized and become effective

Revision of regulations is also much more easily effected than is the amendment of an act of Congress itself. Both of the regulatory documents pertaining to Cytology Laboratories, ( ) Title 42, Part 74 Regulations for Licensure of Clinical Laboratories, and (b) Title 20 Part 405, the Medicare Regulations, are in some stage of revision at present Accordingly we will discuss both the existing regulations pertaining to cytopathology and the proposed changes, insofar as these are developed

Title 42, *Part 74—Regulations for Licensure of* Clinical Laboratories—recognizes Pathology as a diagnostic category separate from Clinical Chemistry Hematology Immunohematology Radiobioassay and Microbiology Pathology is subdivided into three procedures Exfoliative Cytology Histopathology and Oral Pathology The regulations apply to laboratories which accept specimens in interstate commerce, directly or indirectly for the purpose of providing information for the diagnosis, prevention, or treatment of any disease or impairment, or the assessment of the health, of man If laboratory accepts no more than 100 specimens in the entire category of Pathology in any calendar year the general category of Pathology is exempted from regulation. However in practice, exfoliative cytology is licensed as procedure separate and distinct from histopathology and cytopathology laboratories are included in the licensure program if they receive more than 100 cytological specimens yearly in interstate commerce

The subject of Quality Control is dealt with both in general section and in subsection specific for cytology The general section (§74 20) provides that quality controls must include

( ) Preventive maintenance, scheduled inspection, or testing for proper operation of equipment, validation of methods, surveillance of results, and remedy of detected deficiencies, with records showing these.

(b) Physical adequacy of the laboratory its equipment, and methods, for performance of the laboratory procedures for which licensed.

( ) Labeling of reagents as to identity concentration and date evaluation of their reactivity

(d) Immediate availability of current laboratory manuals which completely detail the diagnostic, control and calibration techniques in operation in the laboratory

( ) Dated approval of any changes in techniques

(f) Instruction for specimen collection, preser

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Forum on Criteria for Certification, Licensure and Quality Control of Cytology Laboratories

Held during the re-convened 18th Scientific Meeting of the American Society of Cytology in Honolulu, Hawaii, November 8, 1970

Moderator
  Leopold G. Koss, M.D., Baltimore, Maryland[*]
Participants
  Martin D. Hicklin, M.D., Atlanta, Georgia
  William Kaufmann, M.D., Albany, New York
  Donald W. Penner, M.D., Winnipeg, Canada
  C. P. Schwinn, M.D., Los Angeles, California
  George L. Wied, M.D., Chicago, Illinois

Moderator Koss. The widespread acceptance of cytology as a method of laboratory diagnosis has brought upon us a number of problems having to do with level of laboratory performance. The principal reason for the difficulties that we are about to discuss lies in the fact that barely 25 years elapsed since the value of cytology as a diagnostic weapon has been recognized. The acceptance of cytology by pathologists is still more recent: except for a few individuals and laboratories, massive participation by pathologists dates from 1955 onward. While an effort is being made to train young pathologists in cytology, this effort is still uneven. The older pathologists had to learn cytology on their own, aided by postgraduate courses and seminars.[†]

There has been very little effort at regulating the level of performance in cytology until the Laboratory Improvement Act in 1967 established basic criteria for laboratories engaged in interstate commerce and Medicare services. This Act, which will be discussed *in extenso* during this panel discussion, led the American Society of Cytology to establish a Committee on Licensure which issued a number of certificates to persons without training in pathology who were thereby authorized to practice cytology under the auspices of Medicare and interstate commerce. These certificates were issued either on the strength of past training and experience under the so-called "grandfather clause" or after an examination.

However, there are at this moment no mechanisms either internal or external to the laboratories that would assure us of an adequate level of performance in cytology. Quality control is not extensively practiced, largely because it is very difficult to find an acceptable method. It is virtually certain, however, that the levels of performance in cytology are very variable.

Numerous individuals and professional and Governmental organizations have given considerable thought to the problems. We are indeed very fortunate to have with us this morning a distinguished panel who are going to discuss for us a number of problems having to do with laboratory licensure and quality control. They are:

1. Dr. Martin Hicklin from Atlanta, Georgia. He is the Chief of the Pathology Unit of the Center for Disease Control, Health Services and Mental Health Administration, Public Health Service, U.S. Department of Health, Education and Welfare.
2. Dr. William Kaufmann from Albany, New York. He is the Pathologist Director, Clinical Laboratory Center, New York State Department of Health.
3. Dr. Don W. Penner from Winnipeg, Canada. He is the Director of Laboratories at the Winnipeg General Hospital and Associate Professor of Pathology at the University of Winnipeg. Dr. Penner is here representing the College of American Pathologists.
4. Dr. Charles P. Schwinn from Los Angeles, California. He is the Head of Division of Cytopathology, Los Angeles County Hospital, Associate Professor of Pathology at the University of Southern California Medical School and Chairman, Committee on Cytotechnology, American Society of Clinical Pathologists.[‡]
5. Dr. George L. Wied from Chicago, Illinois. He is the Blum-Riese Professor of Obstetrics and Gynecology and Professor of Pathology at the University of Chicago. Dr. Wied will represent the views of the International Academy of Cytology in his capacity as Chairman of the Committee on Registration and Licensure of the Academy.

Dr. Martin Hicklin will present a summary and critique of Federal Laws and Regulations pertaining to licensure and approval of cytology laboratories of interstate commerce.

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[*] Pathologist-in-Chief, Sinai Hospital of Baltimore, Inc., Professor of Pathology, Thomas Jefferson Medical College.

[†] An initial trial of peer-evaluation and testing has been approved and is being currently implemented (March, 1971).

[‡] Assistant Director, Chief, Laboratory Services, California State Department of Public Health.

Dr. Hicklin:

The current Federal requirements relating to the practice of cytopathology are contained in three documents

1 The Clinical Laboratories Improvement Act of 1967 (CLIA)

2 The Code of Federal Regulations Title 42, Chapter I, part 74 Regulations for Licensure of Clinical Laboratories

3. The Code of Federal Regulations Title 20, Chapter III, Part 405 "Conditions for Coverage of Services of Independent Laboratories, relating to the Health Insurance for the Aged program of the Social Security Administration.

In these various sources the terms cytology and exfoliative cytology are interpreted to mean the examination of scraped, washed, or exfoliated cells, body fluids, or exudates for ( ) detection of cancer possibly pre-cancerous or benign lesions, infections or infestations, (b) monitoring treatment, ( ) screening of normal from non-normal, and (d) the assessment of hormonal activity in the female. For purposes of current licensure cytology does not include cytogenetics, or tissue culture methods for diagnosis of connective tissue or metabolic diseases. Many prefer cytopathology" which will be used synonymously with cytology and exfoliative cytology

Most of the relevant details are contained in the Public Health Service Regulations However it is perhaps in order first to summarize briefly the Clinical Laboratories Improvement Act of 1967 enacted by the 90th Congress Section 5 of Public Law 90-174 amends Part F of Title III of the Public Health Service Act to include clinical laboratories under the licensing authority which previously had extended only to biological products The amendment defines clinical laboratory interstate commerce, and general scope of licensing procedure, with provisions for revocation and redress, exceptions and exemptions, violations and the setting of penalties Cytological examination is included specifically

Essential to the development of our subject is Sec. 353 (d) (1) which, in summary specifies that whatever standards for cytology laboratory operation are devised by the Secretary or his agency must include ( ) *quality control* program, and (b) participation in *proficiency testing program* established by the Department of Health, Education and Welfare in addition to ( ) the professional requirements of the personnel and (d) the physical requirements of the laboratory operation

Federal Regulations are the creation of the executive branch under the broad limits set down by the legislation Federal Regulations are not necessarily formulated by majority vote of deliberative body but wide-ranging expert advice is usually sought in their drafting and diverging views and arguments are invited before they are finalized and become effective

Revision of regulations is also much more easily effected than is the amendment of an act of Congress itself Both of the regulatory documents pertaining to Cytology Laboratories, (a) Title 42, Part 74 Regulations for Licensure of Clinical Laboratories, and (b) Title 20 Part 405, the Medicare Regulations, are in some stage of revision at present. Accordingly we will discuss both the existing regulations pertaining to cytopathology and the proposed changes, insofar as these are developed.

Title 42, Part 74—Regulations for Licensure of Clinical Laboratories—recognizes Pathology as a diagnostic category separate from Clinical Chemistry Hematology Immunohematology Radiobioassay and Microbiology Pathology is subdivided into three procedures Exfoliative Cytology Histopathology and Oral Pathology The regulations apply to laboratories which accept specimens in interstate commerce, directly or indirectly for the purpose of providing information for the diagnosis, prevention, or treatment of any disease or impairment, or the assessment of the health of man If laboratory accepts no more than 100 specimens in the entire category of Pathology in any calendar year the general category of Pathology is exempted from regulation. However in practice, exfoliative cytology is licensed as procedure separate and distinct from histopathology and cytopathology laboratories are included in the licensure program if they receive more than 100 cytological specimens yearly in interstate commerce

The subject of Quality Control is dealt with both in a general section and in subsection specific for cytology The general section (§74 20) provides that quality controls must include

( ) Preventive maintenance, scheduled inspection, or testing for proper operation of equipment, validation of methods, surveillance of results, and remedy of detected deficiencies, with records showing these

(b) Physical adequacy of the laboratory its equipment, and methods, for performance of the laboratory procedures for which licensed

( ) Labeling of reagents as to identity concentration and date evaluation of their reactivity

(d) Immediate availability of current laboratory manuals which completely detail the diagnostic, control and calibration techniques in operation in the laboratory

( ) Dated approval of any changes in techniques.

(f) Instruction for specimen collection, preservation and transportation which will provide accurate and clinically useful results.

Some of the provisions in the *general* section on quality control are not as readily applicable in terms of cytology as they are in terms of chemical or microbiological procedures.

The subsection on Quality Control in Exfoliative Cytology [§74.26 (a)] in effect at the present time provides that the laboratory director or supervisor qualified in cytology or *cytotechnologist rescreen* at least a 10 random sample of the gynecological smears *interpreted as negative or benign* by cytoscreeners and *confirm* all gynecological *smears interpreted as suspicious or positive*. The report is to be signed by a physician qualified in pathology or cytology. All non-gynecological cytological preparations are to be *reviewed* by a director or supervisor qualified in cytology. Mechanical methods should provide quality control similar to those of other nonmanual laboratory procedures. All smears are to be filed for at least two years from date of examination.

In the revised regulations a substitute paragraph has been drafted which contains the following important additions in regard to reports and records.

(a) Unsatisfactory specimens or inadequate case information must be documented in the report to the physician.

(b) The initial screener and the reviewer are to be *identified in the laboratory record kept for* each specimen.

(c) Maintenance *of follow-up records of suspicious* or positive (or the equivalent) smears is mandatory.

(d) *A complete annual report is required* including this information.

In the revision the laboratory report does not have to be *signed* by a physician pathologist or cytologist, but a physician pathologist or cytologist is required to review all smears which are nongynecological and all gynecologic smears which are the equivalent of suspicious or positive. The requirement for rescreening the 10 sample of negative and benign gynecological specimens is retained.

The mechanics of proficiency testing are detailed in §74.40. Proficiency tests are designated in general to assess competency of staff and adequacy *of facilities*, equipment, reagents, procedures, and working conditions for performance of the procedure *for* which licensed. Provision is made that tests may be conducted during on-site inspection or *samples may* be submitted at other times by other means. The regular personnel must examine the test *samples using* the routine method and in the time ordinarily required.

In the proficiency testing regulations there are requirements for determining the performance of applicant laboratories based on the performance of reference and "referee" laboratories *receiving identical* samples. Reference and referee laboratories are further defined in the regulations *in terms of* their role in validating or examining test specimens. *The regulations arbitrarily set the* lower limit of passing performance as within 10 percentage *point of the minimum* grade obtained by the referee laboratories, with the exception of obviously deviant results. *These* criteria again relate clearly to chemistry or microbiology samples, but *in* cytopathology their relevance is obscure.

Admittedly, since identical cytological samples are not generally available, since reference and referee laboratories have not been established yet in cytopathology, and since a universally acceptable numerical grading system has not been devised, no official proficiency test system has been applied to any applicant or licensee laboratory as of this time.

In establishing personnel standards the Clinical Laboratory Regulations refer to the somewhat longer-standing Medicare Regulations, and the interrelationship is rather complicated. There is *some dissatisfaction with these personnel standards* because of ambiguity and loopholes, especially as *they relate to cytopathology*. Effort is being made to correct this, but details are lacking. Eventually *unified personnel standards will probably be established*. Sections 405.1312, 405.1313, and *405.1315 of Title 20, Code of Federal Regulations* (the Medicare Regulations) define and list qualifications *for* laboratory directors, *supervisors, and* technical personnel, respectively. All sections are *applicable* to the interstate licensure program, *with the relatively minor exception that January 1, 1968* is substituted for July 1, 1966 as the time from which qualifying experience is dated.

Section 405.1314 of the Medicare Regulations is applicable without exception, and appears to override the other personnel standards. This section specifies that a laboratory may perform only the procedures and tests which are within the specialities or subspecialities in which the laboratory director is qualified or in which there is a qualified supervisor. *Consequently*, a cytology laboratory may be directed by a physician (a) certified in Clinical *and* Anatomical Pathology by the American Board of Pathology or the American Osteopathic Board of Pathology, or eligible for certification, (b) certified or eligible in anatomical pathology *or* clinical pathology, or (c) certified by the American Society of Cytology, or possessing qualifications equivalent to those required for certification. Finally, a cytology laboratory may be directed by

" ... an individual *who has* demonstrated competency through at least 7 years of accumulated experience in a position of diagnostic responsibility in the field of clinical cytology or through 5 years of full time training in diagnostic clinical cytology with suitable endorsement by a physician supervisor and by the publishing of treatises, texts, and other publications on the subject of exfoliative cytology which are generally acknowledged and recognized by the medical profession as authoritative in the field by his appointment to and service in pertinent teaching and research positions in recognized schools of medicine by his acceptance into or award of membership and office in professional societies in this field and by his receipt of other professional honors for excellence in the use of procedures in exfoliative cytology for the diagnosis of a pathological condition.

As an additional statement, depending on the size and function of the laboratory the [qualified] laboratory director may also serve as the laboratory supervisor.

If the person designated as director does not meet any of these qualifications, for example if the applicant laboratory combines cytopathology with other categories or procedures and the laboratory director is qualified because of competence or certification in one of the other categories but *not* in cytology then the *supervisor* of the cytopathology service must possess one or another of the special qualifications that were required in the foregoing of the laboratory director.

A person qualified as general supervisor is required to be on the premises all of the time in which cytological screening is being performed, although the director is not specifically required to be. If the director is qualified in cytology according to one or another of the criteria, the general supervisor in cytology may actually be a medical technologist. This is one of the areas in which there is dissatisfaction with the interpretation of the regulations. The Medicare Regulations do not recognize the special training and experience which are necessary for competence in cytotechnology as compared with the general clinical laboratory training and experience leading to qualification in medical technology. To make up this deficiency the detailed qualifications of a cytotechnologist are stated in Section 74.31(j) of the Clinical Laboratory Regulations.

Unfortunate as it may seem, nowhere is the statement made that cytoscreeners have to be cytotechnologists. In Medicare Regulations Section 403.1313, the term clinical laboratory technologist is defined and qualifications are listed. These personnel are designated to proficiently perform under general supervision the clinical laboratory tests which require the exercise of independent judgment and responsibility. No special reference is made to cytology in this context, however, and under the regulations as they are interpreted at present anybody can screen smears in a cytology laboratory under the supervision or direction of a qualified person. The present quality control regulations provide that the rescreener or reviewer of the 10 sample be a qualified director or supervisor or at least meet the requirements of the cytotechnologist as defined. In the proposed revision of the quality control regulations the rescreener must be at least a supervisory cytotechnologist, which is as yet undefined.

General Provisions are contained in Subpart G and deal with the handling of records and reports, maintenance of equipment and facilities, inspection, and change in ownership or key personnel.

It is required that records of observation be made concurrently with each step in the examination, kept at least two years after the diagnostic report is sent out, and available for inspection.

Personnel records must be current and show each employee's training, experience, duties, and dates of employment.

Specimen accession records are to include an identifying number, name and other identification of the person from whom submitted, if available, the name of the physician, person or laboratory submitting the specimen, the date collected and the date received in the laboratory, the condition, if unsatisfactory, the test performed and the result and date of completion.

The laboratory report is to be sent promptly to the submitting physician or other person, and a copy is to be retained at least two years. Cytodiagnoses are required to be in established nomenclature. The general provisions are somewhat repetitious of the general subsection on Quality Control, restating that space, facilities, and equipment are to be adequate and free from hazard, and referring again to the equipment maintenance program.

The federal agency responsible for enforcement of the CLIA is given great leeway in the content and frequency of reports required of applicants and licensees. The regulations are also rather broad concerning inspections, which must be made at least annually and otherwise from time to time.

In practice, applicants are asked initially to submit fairly detailed reports regarding directory, supervisory, technological, and cytotechnological personnel only. Requests for additional personnel information are the result of awareness in the initial reporting of qualifications. Renewal applications include gains and losses of personnel in these categories.

Applicants are not required to fill out reports listing categories of tests performed, volume, techniques used, equipment and facilities, and quality control programs. Details regarding these are obtained by the government examiners during vation, and transportation which will provide accurate *and* clinically useful results.

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In the revised regulations a substitute paragraph has been drafted which contains the following important additions in regard to reports and records:

(a) Unsatisfactory specimens or inadequate case information must be documented in the report to the physician.

(b) The initial screener and the reviewer are to be identified in the laboratory record kept for each specimen.

(c) Maintenance of follow-up records of suspicious or positive (or the equivalent) smears is mandatory.

(d) A complete annual report is required, including this information.

In the *revision the laboratory report does not* have to be *signed* by a physician pathologist or cytologist, but a physician pathologist or cytologist is required to *review* all smears which are non-gynecological, and all gynecologic smears which are the equivalent of suspicious or positive. The requirement for rescreening the 10 sample of negative and *benign gynecological specimens* is retained.

The mechanics of proficiency testing are detailed in §74.40. Proficiency tests are designated in general to assess competency of staff and adequacy of facilities, equipment, reagents, procedures and working conditions for performance of the procedure for which licensed. *Provision is made that* tests may be conducted during on-site inspection *or samples* may be submitted at other times by other means. The regular personnel must examine the test samples using the routine method, and in the *time ordinarily* required.

In the proficiency testing regulations there are requirements *for determining* the performance of applicant laboratories based on the performance of reference and "referee" laboratories receiving *identical* samples. Reference and referee laboratories are *further* defined in the regulations in terms of their role in validating or examining test specimens. *The regulations arbitrarily set the lower* limit of passing performance as within 10 percentage points of the minimum grade obtained by the referee laboratories, with the exception of obviously deviant results. These criteria again relate clearly to chemistry or microbiology samples, but in cytopathology their relevance is obscure.

Admittedly, since *identical* cytological samples are not generally available, since reference and referee laboratories have *not been established yet* in cytopathology, and since a universally acceptable numerical grading system has not been devised, no official proficiency test system has been applied to any applicant or licensee laboratory as of this time.

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labor in the field of clinical cytology or through 5 years of full time training in diagnostic clinical cytology with suitable endorsement by a physician supervisor and by the publishing of treatises, texts, and other publications on the subject of exfoliative cytology which are generally acknowledged and recognized by the medical profession as authoritative In the field by his appointment to and service in pertinent teaching and research positions in recognized schools of medicine by his acceptance into or award of membership and office in professional societies in this field and by his receipt of other professional honors for excellence in the use of procedures in exfoliative cytology for the diagnosis of pathological condition

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In practice, applicants are asked initially to submit fairly detailed reports regarding directory supervisory technological, and cytotechnological personnel only Requests for additional personnel information are the result of vagueness in the initial reporting of qualifications Renewal applications include gains and losses of personnel in these categories.

Applicants are not required to fill out reports listing categories of tests performed, volume, techniques used, equipment and facilities, and quality control programs. Details regarding these are obtained by the government examiners during proper work manuals and the maintenance of safety precautions.

The Department has gained considerable experience in the operation of this proficiency testing program which was primarily designed to be educational, although enforcement policies had to be established because evidence had been accumulating prior to the enactment of legislation, that laboratory performance in the State was not what it should have been. The need for improvement in quality performance was amply demonstrated in the course of the proficiency testing program and there were rather marked deficiencies in chemistry and bacteriology (1). In fact, within the first three years of the program, approximately 50 laboratories out of a total of 400 operating in the State of New York, and outside of the City of New York which has its own clinical laboratory act, closed their doors. Only 3 such laboratories had to be forced to cease operations by Departmental actions while 47 decided to close voluntarily for reasons of their own.

Since clinical laboratory supervision in the State was actually the first step in the government's assumption of guaranteeing adequate delivery of health care to its citizens, it was essential that the Department proceed slowly and deliberately in such areas where scientific and responsible supervision was possible. Therefore the Department limited its activities of supervision, education and enforcement to the disciplines of clinical laboratory practice. The Division of Laboratories and Research of the New York State Department of Health had decades of experience with a program of proficiency in pathologic anatomy in which all pathologists who wished to assume directorship of an approved laboratory in the State again with the exception of New York City participated. This demonstration of proficiency in pathology was abolished as such with the enactment of the Laboratory Services Act of 1964 at which time certification of pathologist by the American Board of Pathology replaced the previously required test. While the new law specifically required that all laboratories be directed by individuals holding a certificate of qualification it also stipulated that they continuously demonstrate their ability to perform. This included the disciplines of pathology and cytology.

Therefore in 1968 the Department embarked on the first computerized program of proficiency testing in diagnostic cytology. The purposes were to evaluate the performance of cytotechnologists, to determine any relationship of performance to a variety of laboratory factors, and to relate results and data to the educational background of cytotechnologists thus offering the possibility for the establishment of standards applicable to laboratories performing diagnostic exfoliative cytology.

Test specimens consisting of 3 cervical-vaginal and 2 sputum smears were carried by our clinical laboratory investigators to each laboratory holding a permit in exfoliative cytology to be tested on site by the cytotechnologist routinely performing this task. Whereas an ideal testing program should include smears from all body fluids initial limitation to most frequently examined material was considered desirable. With the help of the Office of Electronic Data Processing of the New York State Department of Health, a data processing program was formulated before testing.

It was hoped that such a program would allow for the accumulation of data for baseline information on performance and education of cytotechnologists and the tabulation of characteristics of cytology laboratories. I regret that the lack of proper projection equipment prevents me from illustrating to you the details of this program. However as Doctor Wied informs me the paper which was submitted to *Acta Cytologica* on the subject and which contains detailed information in the form of figures and tables will appear in print very shortly.

Let me say though, that selection of the smears for proficiency testing was made in our laboratory where at least two pathologists and sometimes three examined the smears. This selection was conditional on complete agreement of interpretation. Three additional laboratories, namely the Memorial Hospital for Cancer and Allied Diseases in New York City, the Albany Medical College of Union University in Albany and the Crouse Irving Memorial Hospital in Syracuse were chosen as reference laboratories. Each of these laboratories examines specimens in excess of 15 000 annually. Agreement on the interpretation of the smears by all three reference laboratories was a prerequisite as was confirmation by biopsy of all except negative specimens.

Rating of test specimens by each cytotechnologist in a laboratory was based on 20 points per smear and total correct identification of all smears equalled 100 points. A passing grade of 80 points was established thus allowing one 20 point error or two 10 point errors in the interpretation of the five smears. The cytotechnologist test form was accompanied by an instruction sheet. The instruction sheet was sufficiently detailed to eliminate misunderstanding of the final reporting and the laboratory investigator was also present to explain any possible ambiguity. Identification and cross reference were insured by use of the laboratory number in the upper right hand corner of the test form as well as by the cytotechnologist's Social Security number.

A report of the cytotechnologist's test was sent to the director of the cytology laboratory. All errors were tabulated against the correct answers and comments were added to serve as guides for the cytotechnologist and the director of the laboratory. Failure to achieve 80 points made retesting mandatory. If the second testing results were still unsatisfactory, third test was required.

A cytology personnel questionnaire was developed to determine the possible relationship of past and present education, training, and experience to the cytotechnologist's performance. Since scope of work in the laboratory, methodology, laboratory procedures (such as Papanicolaou staining and acridine orange fluorescence technics) in addition to supervision by pathologists, may also reflect on performance, a form was designed to obtain this information as well as type and degree of supervision by the pathologist, availability of follow-up data, and workload of each laboratory.

The clinical laboratory proficiency testing program in other disciplines requires our laboratory investigators to transcribe fairly detailed narrative report of their observations of physical facilities, work condition, type and quality of equipment, availability of teaching material, and record keeping in the laboratories. Therefore similar report was requested about laboratories engaged in diagnostic cytology for evaluation of general standards. Additional information was included in the general narrative report filed after completion of testing and inspection of other laboratory categories.

As an adjunct to the proficiency testing program, the teaching Kodachrome of the Month series was initiated and this is now in its second year. Interesting and informative cases were distributed to all laboratories with a permit in exfoliative cytology. These cases, consisting of one or more photographed fields from smears, were accompanied by histories, discussions and references.

In 1968 there were 182 laboratories with permits in cytology. During the initial six-month period of the program 130 laboratories were inspected and 111 cytotechnologists were tested. Sixty-eight laboratories visited employed no cytotechnologists (all screening being done by the pathologist) and 62 employed one or more cytotechnologists. Eighty-six cytotechnologists achieved satisfactory grade, 40 received perfect score and 46 showed varying combinations of errors. Of the 111 cytotechnologists participating in this initial testing 25 failed to attain a satisfactory grade.

At this time are in the process of revising our approach as result of experience with the program as well as advice from our expert advisory committee. A larger number of laboratories and more cytotechnologists are presently participating. We fully recognize that our approach is experimental, that more experience will have to be gained with variety of methods, that an external quality control program such as this proficiency testing program is only part of overall laboratory proficiency. It has been argued that our program tests the cytotechnologist and not the proficiency of the laboratory. While this may well be so, it is difficult to see how the field of exfoliative cytology anyone or anything but the cytotechnologist can be tested. It is he or she on whom the proficiency of the laboratory primarily depends, even if cytotechnologists are used only as screeners.

It would appear that the rapidly changing atmosphere of the delivery of health care where the concept of the *right* of the citizen to adequate medical care has replaced the old tenet of the *privilege* of medical care we have to accept the entrance of regulating agencies into the delivery of health care. Whether this good or bad" only time will tell, but our responsibility physicians and scientists is to lend helping hand to these agencies if it were for nothing else but for guidance. Cooperation and collaboration between government and independent scientific organizations should be an important step this direction.

Dr. Hicklin so aptly demonstrated in his exposé on the Federal program this morning.

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Kaufmann, W., Kelly, J., Vanderlinde, R., Fasce, C., Gordon, M., Stevens, R., Graf, H.: Clinical Laboratory Performance: Experience with New York State Clinical Laboratory Program. New York State J. Med. 69: 198 (July) 1 69.

Moderator Kove: Dr. Don W. Penner will present the of the Subcommittee on Quality Control in Histopathology and Cytology of the Committee on Standards of the College of American Pathologists.

Dr. Penner:

Organized Laboratory Medicine, in contrast to many other segments of medicine, has been very concerned, and as result actively involved in the improvement of the quality of their service. One has only to look at the activities of the College of American Pathologists and the American Society of Clinical Pathologists. The involvement in ongoing education, quality of performance evaluation and laterally the instigation of the Inspection and Accreditation Program and the most recent developing Quality Assurance Service of the College attests to the tremendous interest and effort to achieve excellence.

In the past most of our efforts has been directed to Clinical Pathology to insure that the data we generate is reliably accurate. In Anatomical Pathology and Cytology we have, for many years, conducted slide seminars and provided numerous short courses in histopathology. There are also proper work manuals, and the maintenance of safety precautions.

The Department has gained considerable experience in the operation of this proficiency testing program which was primarily designed to be educational although enforcement policies had to be established because evidence had been accumulating prior to the enactment of legislation that laboratory performance in the State was not what it should have been. The need for improvement in quality performance was amply demonstrated in the course of the proficiency testing program and there were rather marked deficiencies in chemistry and bacteriology. In fact, within the first three years of the program, approximately 50 laboratories out of a total of 400 operating in the State of New York, and outside of the City of New York which has its own clinical laboratory act, closed their doors. Only 3 such laboratories had to be forced to cease operations by Departmental actions, while 47 decided to close voluntarily for reasons of their own.

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Test specimens consisting of 3 cervical-vaginal and 2 sputum smears were carried by our clinical laboratory investigators to each laboratory holding a permit in exfoliative cytology to be tested on the by the cytotechnologist routinely performing this task. Whereas an ideal testing program should include smears from all body fluids, initial limitation to most frequently examined material was considered desirable. With the help of the Office of Electronic Data Processing of the New York State Department of Health, a data processing program was formulated before testing.

It was hoped that such a program would allow for the accumulation of data for baseline information on performance and education of cytotechnologists and the tabulation of characteristics of cytology laboratories. I regret that the lack of proper projection equipment prevents me from illustrating to you the details of this program. However as Doctor Wied informs me, the paper which was submitted to *Acta Cytologica* on the subject and which contains detailed information in the form of figures and tables will appear in print very shortly.

Let me say though, that selection of the smears for proficiency testing was made in our laboratory where at least two pathologists and sometimes three examined the smears. This selection was conditional on complete agreement of interpretation. Three additional laboratories, namely the Memorial Hospital for Cancer and Allied Diseases in New York City, the Albany Medical College of Union University in Albany and the Crouse-Irving Memorial Hospital in Syracuse were chosen as reference laboratories. Each of these laboratories examines specimens in excess of 15 000 annually. Agreement on the interpretation of the smears by all three reference laboratories was a prerequisite as was confirmation by biopsy of all except negative specimens.

Rating of test specimens by each cytotechnologist in a laboratory was based on 20 points per smear and total correct identification of all smears equalled 100 points. A passing grade of 80 points was established thus allowing one "20" point error or two "10" point errors in the interpretation of the five smears. The cytotechnologist test form was accompanied by an instruction sheet. This instruction sheet was sufficiently detailed to eliminate misunderstanding of the final reporting and the laboratory investigator was also present to explain any possible ambiguity. Identification and cross reference were insured by use of the laboratory number in the upper right hand corner of the test form as well as by the cytotechnologist's Social Security number.

A report of the cytotechnologist's test was sent to the director of the cytology laboratory. All errors were tabulated against the correct answers, and comments were added to serve as guides for the cytotechnologist and the director of the laboratory. Failure to achieve 80 points made retesting mandatory. If the second testing results were still unsatisfactory a third test was required.

A cytology personnel questionnaire was developed to determine the possible relationship of past and present education, training and experience on the cytotechnologist performance. Since scope of work in the laboratory methodology laboratory procedures (such as Papanicolaou staining and acridine orange fluorescence technics) in addition to supervision by pathologists, may also reflect on performance, a form was designed to obtain this information as well as type and degree of supervision by the pathologist, availability of follow up data, and workload of each laboratory.

The clinical laboratory proficiency testing program in other disciplines requires our laboratory investigators to transcribe a fairly detailed narrative report of their observations of physical facilities, work condition, type and quality of equipment, availability of teaching material, and record keeping in the laboratories. Therefore a similar report was requested about laboratories engaged in diagnostic cytology for evaluation of general standards. Additional information was included in the general narrative report filed after completion of testing and inspection of other laboratory categories.

As an adjunct to the proficiency testing program, the teaching Kodachrome of the Month series was initiated and this is now in its second year. Interesting and informative cases were distributed to all laboratories with permit in exfoliative cytology. These cases, consisting of one or more photographed fields from smears, were accompanied by histories, discussions and references.

In 1968 there were 182 laboratories with permits in cytology. During the initial six-month period of the program 150 laboratories were inspected and 111 cytotechnologists were tested. Sixty-eight laboratories visited employed no cytotechnologists (all screening being done by the pathologist) and 82 employed one or more cytotechnologists. Eighty six cytotechnologists achieved a satisfactory grade, 40 received a perfect score and 46 showed varying combinations of errors. Of the 111 cytotechnologists participating in this initial testing 25 failed to attain a satisfactory grade.

At this time we are in the process of revising our approach as a result of experience with the program as well as advice from our expert advisory committee. A larger number of laboratories and more cytotechnologists are presently participating. We fully recognize that our approach is experimental, that more experience will have to be gained with a variety of methods, that an external quality con tory proficiency. It has been argued that our program tests the cytotechnologist and not the proficiency of the laboratory. While this may well be so, it is difficult to see how in the field of exfoliative cytology anyone or anything but the cytotechnologist can be tested. It is he or she on whom the proficiency of the laboratory primarily depends even if cytotechnologists are used only as screeners.

It would appear that in the rapidly changing atmosphere of the delivery of health care where the concept of the *right* of the citizen to adequate medical care has replaced the old tenet of the *privilege* of medical care we have to accept the entrance of regulating agencies to the delivery of health care. Whether this is "good or bad" only time will tell, but our responsibility as physicians and scientists is to lend a helping hand to these agencies if it were for nothing else but for guidance. Cooperation and collaboration between government and independent scientific organizations should be an important step in this direction as Dr Hicklin so aptly demonstrated in his exposé on the Federal program this morning.

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2 Kaufmann, W Kelly J Vanderlinde R Fasce C Gordon, M Stevens, R Graft, H Clinical Laboratory Performance Experience with New York State Clinical Laboratory Program New York State J Med 69 1969 (July) 1969

Moderator Koss: Dr Don W Penner will present the views of the Subcommittee on Quality Control in histopathology and cytology of the Committee on Standards of the College of American Pathologists.

Dr Penner:

Organized Laboratory Medicine in contrast to many other segments of medicine has been very concerned, and as a result actively involved in the improvement of the quality of their service. One has only to look at the activities of the College of American Pathologists and the American Society of Clinical Pathologists. The involvement in ongoing education, quality of performance evaluation and laterally the instigation of the Inspection and Accreditation Program and the most recent developing Quality Assurance Service of the College attests to the tremendous interest and effort to achieve excellence.

In the past most of our efforts have been directed to Clinical Pathology to insure that the data we generate is reliably accurate. In Anatomical Pathology and Cytology we have for many years, proper work manuals, and the maintenance of safety precautions.

The Department has gained considerable experience in the operation of this proficiency testing program which was primarily designed to be educational although enforcement policies had to be established because evidence had been accumulating prior to the enactment of legislation, that laboratory performance in the State was not what it should have been. The need for improvement in quality performance was amply demonstrated in the course of the proficiency testing program and there were rather marked deficiencies in chemistry and bacteriology.[4] In fact, within the first three years of the program, approximately 50 laboratories out of a total of 400 operating in the State of New York, and outside of the City of New York which has its own clinical laboratory act, closed their doors. Only 3 such laboratories had to be forced to cease operations by Departmental actions, while 47 decided to close voluntarily for reasons of their own.

Since clinical laboratory supervision in the State was actually the first step in the government's assumption of guaranteeing adequate delivery of health care to its citizens, it was essential that the Department proceed slowly and deliberately in such areas where scientific and responsible supervision was possible. Therefore the Department limited its activities of supervision, education and enforcement to the disciplines of clinical laboratory practice. The Division of Laboratories and Research of the New York State Department of Health had decades of experience with a program of proficiency in pathologic anatomy in which all pathologists who wished to assume directorship of an approved laboratory in the State, again with the exception of New York City, participated. This demonstration of proficiency in pathology was abolished as such with the enactment of the Laboratory Services Act of 1964 at which time certification of pathologists by the American Board of Pathology replaced the previously required test. While the new law specifically required that all laboratories be directed by individuals holding a certificate of qualification, it also stipulated that they continuously demonstrate their ability to perform. This included the disciplines of pathology and cytology.

Therefore in 1968 the Department embarked on the first computerized program of proficiency testing in diagnostic cytology. The purposes were to evaluate the performance of cytotechnologists, to determine any relationship of performance to a variety of laboratory factors, and to relate results and data to the educational background of cytotechnologists thus offering the possibility for the establishment of standards applicable to laboratories performing diagnostic exfoliative cytology.

Test specimens consisting of 3 cervical-vaginal and 2 sputum smears were carried by our clinical laboratory investigators to each laboratory holding a permit in exfoliative cytology, to be tested on site by the cytotechnologist routinely performing this task. Whereas an ideal testing program should include smears from all body fluids, initial limitation to most frequently examined material was considered desirable. With the help of the Office of Electronic Data Processing of the New York State Department of Health, a data processing program was formulated before testing.

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A report of the cytotechnologist's test was sent to the director of the cytology laboratory. All errors were tabulated against the correct answers, and comments were added to serve as guides for

Medicare and Medi-Cal patients. Normally, following the report of an Advisory Ad Hoc Committee (see report from Doctor Bodily to the Ad Hoc Advisory Committee on Standards above) the Department of Public Health invites all interested parties to comment on the proposed regulations. Interested parties in this instance might include individual cytotechnologists, individual cytopathologists, organizations whose membership included either cytotechnologists, cytopathologists, or both. It might include operators of private laboratories either within or without the State of California, and it might include medical laboratory technologists. Since I had personally discussed the Committee meeting with the members of the California Association of Cytotechnologists, considerable interest concerning the proposed regulations was engendered. There resulted an article in the Cytotechnologist Bulletin, Volume VI, Number 2, 1969 entitled "Licensure for Cytotechnologists" by Rene Dougherty, C.T. (ASCP) as follows: "Although there had been occasional discussions among members of the California Association of Cytotechnologists about the desirability of legal and professional recognition of their specialty by the state, the Association had made no statement of policy regarding licensure, nor made any attempt to obtain legislation toward it, until the advent of the Medicare program.

At present, California state codes deal only with the directors of laboratories making exfoliative cytologic diagnoses. Cytotechnologists, though not mentioned as a separate entity, are considered to be "screeners," as defined in the Laboratory Act section on medical technologists. In an effort to draft state legislation to conform to the U.S. Department of Health, Education and Welfare Social Security Administration regulations concerning the operation of clinical laboratories performing cytologic tests, and the qualifications of their personnel, an Ad Hoc Advisory Committee was constituted in 1967 by Howard L. Bodily, Ph.D., Chief, Division of Laboratories, State of California Department of Public Health.

"To serve on this Committee, Dr. Bodily selected from various areas of the state several cytopathologists who are in charge of AMA-accredited cytology teaching institutions and/or engaged in diagnostic clinical cytology. Among those chosen were two of CAC's past Pathologist Advisers, William H. Kern, M.D., Director, School of Cytology, Hospital of the Good Samaritan Medical Center, Los Angeles, and Charles P. Schwinn, M.D., Director, School of Cytology, Los Angeles County University of Southern California Medical Center. Before attending the first session scheduled for the purpose of outlining legislative protocols, they arranged to meet with the CAC Board of Directors to discuss the responsibilities, and limitations of cytotechnologists, and, accordingly, made recommendations to the Committee.

It was decided that, for the present, fulfillment of the ASCP Registry requirements (the standards set forth by the Social Security Administration) would be acceptable for state licensure. *However, there is considerable reservation about whether, with proliferating investigative methods and increasing sophistication of instrumentation in the field, the current ASCP criteria will insure the utmost competence of cytotechnologists-of-the-future.* Many CAC members feel that cytotechnology should eventually be put on a degree basis, and that a B.A. be a prerequisite for certification. *Meanwhile the proposed legislation, in making state licensure requirements reciprocal with those of the national Registry, would allow any ASCP registered or eligible cytotechnologists to obtain employment in California without being subjected to a state Board examination.* The tentative bill also includes a "grandfather" clause to protect the positions of those who have been practicing cytotechnologists prior to the date of passage of such legislation. Quality control would be maintained in a stipulation that all cytotechnologists much work for a qualified cytopathologist in an approved laboratory as designated by the Social Security Administration.

As constituted, the bill has been temporarily shelved due to certain conflicts with existing codes, but licensure appears to be imminent and the majority of CAC members are enthusiastic about the prospect of securing state certification. They believe that cytotechnologists as a group will benefit from the attainment of professional status; that, concomitantly, the individual cytotechnologist may expect improvement in working conditions and elevation of salaries, and that the community at large will be better served through regulation of cytology laboratory personnel.

"Toward these goals, CAC has adopted several measures. So far our only direct communication with the Ad Hod Committee has been a *letter from our President to its Chairman, stating that CAC goes on record as approving state licensure but opposes any legislation that would restrict the place where screening may be done; the cytotechnologist should be allowed to screen wherever is mutually agreeable to him and his employer, provided that said employer is a qualified cytopathologist. In reply, the Chairman assured CAC that we would be notified of any future meetings of the Committee so that we may have a representative attend; our attorney has also offered to be present to give necessary legal advice.*

"CAC is now in the process of chartering chapters in San Diego, Los Angeles, and the Bay and South Bay Areas of San Francisco. To accommodate [illegible] physician who is certified by the American Society of Cytology to practice cytopathology or possesses qualifications which are equivalent to those required for certification and the laboratory performs such tests only on that anatomic site for which he is certified, or (iii) is an individual who has demonstrated competency (a) through at least 7 years of accumulative experience in a position of diagnostic responsibility in the field of clinical cytology or through 5 years of fulltime training in diagnostic clinical cytology with suitable endorsement by a physician who has been his supervisor in such activity and (b) by the publishing of treatises, texts and other publications on the subject of exfoliative cytology which are generally acknowledged and recognized by the medical profession as authoritative in the field by his appointment to and service in pertinent teaching and research positions in recognized schools of medicine by his acceptance into or award of membership and office in professional societies in this field and by his receipt of other professional honors for excellence in the use of procedures in exfoliatiave cytology for the diagnosis of a pathological condition and the laboratory performs such tests only on that anatomic site with respect to which such competency is so established The Social Security Administration, after documentation of the individual's qualification by the State agency will, with appropriate professional advice from the Public Health Service make all determinations with respect to the requirements set forth in (iii) of this subparagraph The individual who qualifies under (ii) and (iii) of this subparagraph is deemed also to meet the requirements of 405 1317(b) (2) (ii) of this Subpart M

“Factor 8 If the factor in subparagraph (1) or (6) of this paragraph is not met and the laboratory performs tests in oral pathology the director or a supervisor is a dentist who is certified in oral pathology by the American Board of Oral Pathology or possesses qualifications which are equivalent to those required for certification (board eligible)

2 Cytology Technologist—The Conditions for Converage of Services of Independent Laboratories do not specify the requirements for cytology technologists. Therefore, the following standards will apply for California

“Cytology technologists and clinical laboratory technologists engaged in cytology examinations must work under the direct and responsible supervision of a qualified laboratory director as defined above

“A cytology technologist must meet the following requirements in order for the laboratory to be approved for the Medicare or Medi-Cal Program

Two years of college including 12 semester or 18 quarter hours of science of which 8 semester or 12 quarter hours are in biological science and twelve months of training in a school of cytotechnology approved by the California State Department of Public Health and the Council on Medical Education of the American Medical Association.

Persons who have engaged in cytotechnology since January 1 1967 may be licensed upon successful completion of a written and practical examination provided, however that if an individual is certified as a cytotechnologist by the American Society of Clinical Pathologists, he may be licensed without examination ”

State Department of Public Health, February 15 1968

You will note, in Paragraph 2 of the memorandum from Doctor Bodily to the Ad Hoc Advisory Committee that he states, we have been advised by the Attorney General's representative that licensure of cytology technologists is not possible under the present wording of the California Business and Professions Code Doctor Bodily then called attention to the fact that there was a possibility that the clinical laboratory laws in the State of California might be revised in the near future In actual fact, the proposed revision, in laws relating to clinical laboratories under the California Business and Professions Code had been under serious and continuous revision for the two to three years prior to the meeting of our committee It had been assumed that these revision would be adopted sometime early in 1969 For a variety of reasons however these revisions have not yet been adopted (January 15 1971) These proposed revisions would have allowed the State Department of Health to write regulations concerning licensure of cytology technologist and the licensure of cytology laboratories

As explained to me law relating to pathology laboratories, following passage by the legislature is established in the Business and Profession Code There is a special section of the Business and Professions Code which relates to the clinical laboratories Regulations to define the law and to permit enforcement of the law must be promulgated by the Board of Health of the State of California This is done through the California State Department of Public Health Division of Laboratories Doctor Bodily is Chief of the Division of Laboratories The Ad Hoc Advisory Committee formed by the Department of Public Health under the chairmanship of Doctor Bodily was originally convened to consider requirements for cytology laboratories and cytotechnologists with respect to and will conform to the following quality control standards set forth by the Academy

1) The laboratory is required in its final report in every case to document possible inadequacies of the specimen preparation (such as improper preparation or poor fixation) or inadequacies of clinical information. The minimum information must include: anatomic site of preparation on *every* specimen, the age of the patient, previous therapy if any (endocrine or irradiation) pregnancy status, if any and last menstrual history (on material from the female reproductive tract)

2) A pathologist, physician-cytologist or supervisory cytotechnologist shall rescreen for proper staining and correct diagnostic interpretation, at least ten per cent (10%) random sample of cytologic specimens from the female reproductive tract which have been interpreted as negative "benign by personnel, other than the director or supervisor In the sense of this statement, supervisory cytotechnologist is cytotechnologist who has had more than five (5) years of full-time experience in cytotechnology

3) *All* cytologic specimens interpreted as suspicious" or "positive and at least fifteen per cent (15%) of the cytologic preparations from anatomic sites other than the female genital tract, interpreted as "negative by the prescreening cytotechnologist, shall be reviewed by pathologist or physician-cytologist whose identity shall be documented on the final report Another fifteen per cent (15%) of these cytologic preparations shall be rescreened by supervisory cytotechnologist who must have had at least ten (10) years of full-time experience in cytotechnology If such person is not available in the laboratory the pathologist or physician-cytologist shall review at least thirty per cent (30%) of the material originally identified as negative" by the prescreening cytotechnologist.

4) The laboratory shall request from clinicians, and then properly catalogue follow-up information on all "suspicious" or positive" smears

5) The laboratory shall prepare annual reports which shall include number of cases screened in each category origin of smears, corroborating follow-up data, and discrepancies, if any between clinical data, tissue sections and cytologic findings.

6) The laboratory shall retain all cytologic specimens for period of at least three (3) years from the date of examination.

7) Realizing that continuing education is essential for quality control, the laboratory shall be required to provide regularly scheduled educational sessions for the benefit of the staff members A minimum of one (1) hour per week should be devoted to continuing education.

8) If certified by the Academy it is understood that the Certification is valid only for three (3) trol Standards of the Academy shall be displayed with the Certificate of Registration in prominent place in the laboratory

Details Required for the Site Visit

The following documentations and details are required for the site visit by the representative( ) of the INTERNATIONAL ACADEMY OF CYTOLOGY

1) Patient file book or registry for the past three years,

2) Copies of correspondence with clinicians related to follow-up procedures on patients on whom atypia were cytologically detected

3) Listings of the daily work-load accomplished by each of the cytotechnologists during the past three months;

4) Demonstration of continuing education session as stipulated in Point #7 of the above quality control standards

5) The annual report of the past year as stipulated in Point #5 of the above quality control standards

The above material or details will be made available to the site visitor( ) and the Director Assistant Director Associates, and Chief-Cytotechnologist will be available for interview The site visitor( ) will also be given the opportunity to interview the other cytotechnologists and staff members

Information to Applicant Laboratory About the Procedure to be Expected

1) After receipt of the application, the applicant laboratory will obtain notification that the application is either complete, or requires additional details,

2) The application is then circulated among the Members of the Committee on Registration and Licensure and applicant laboratory will be informed if the application is *Accepted* If application is accepted, the applicant laboratory will receive notification that the fee may be paid The fee for Fellows and Members of the International Academy of cytology is $50.00 (fifty dollars U S currency) The fee for others is $300.00 (three hundred dollars)

3) Special arrangements may be made for applicants in countries from which transfers of U S currency is legally impossible

4) After receipt of the fee (or after special arrangements were made) the data of the site visit will be set,

5) The site visit determines whether or not the laboratory may be certified No refunds will be made, if certification is impossible, but another site visit will be

on Legislation to be composed of members from each chapter who will keep CAC apprised of any legislation state or Federal which would affect the practice of cytotechnology In the interim, a Special Committee has been appointed to study all aspects of licensure and make recommendations to the membership

Thus, with its growing numbers contributing valuable suggestions and support, the California Association of Cytotechnologists may anticipate having a strong voice in determining the course of pertinent legislation."

Unfortunately the proposed revisions in the laws relating to clinical laboratories under the California Business and Professions Code which would have enabled the Department of Public Health to write regulations concerning standards for cytology technologists and cytology laboratories were not acted upon by the State legislature for a variety of political reasons. In fact, they have not been acted upon to this date (January 15 1971) However during the year 1970 the legislature did enact the following change in the Business and Professions Code

"Article 9 Section 1270 is added to the Business and Professions Code to read

"1270 The board shall establish by regulation the limited laboratory activities in which cytotechnologists may engage and shall establish the extent of supervision required and the minimum qualifications to be met by such persons Persons engaged in such activities shall do so only under the supervision of a licensed technologist or person duly authorized to direct a laboratory who is functioning as a technologist. Before such persons are permitted to perform tests, they shall satisfactorily demonstrate their ability to do so in ccordance with procedures prescribed by the epartment"

Signed by Governor 9-14-70

Although the wording of this legislative act may m somewhat obscure to a registered cytotech ogist I have been assured by Doctor Bodily t this revision of the Business and Professions e enables the Department of Public Health rite regulations on standards for cytology ologists and cytology laboratories In recent conversations with Doctor Bodily I have been assured that this matter will be acted upon in the near future It is hoped that the regulations will be similar to those proposed by initial Ad Hoc Advisory Committee on Standards for Cytology Technologists. Furthermore the federal and other state regulations as outlined by Doctor Hicklin[§] and Doctor Kaufmann are now available and furnish further guidelines for consideration.

In assessing the cytotechnologists position in California, one must remember that in California, medical laboratory technologists have been licensed for many years. The licensing requirements for medical technologists in California exceed those required for certification as a medical technologist by the American Society of Clinical Pathologists. This may account in part for the peculiar wording of the revision in the business and Professions Code cited above, since cytotechnologists at the present time are not licensed and therefore, not specifically recognized in the regulations.

Moderator Koss: Dr Wied will discuss for us the efforts of the International Academy of Cytology to standardize the performance of the cytology laboratories throughout the world. Dr Wied.

The INTERNATIONAL ACADEMY OF CYTOLOGY (Committee on Registration and Licensure) requires for Certification of laboratories of diagnostic cytology that the laboratories do

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[§] Of more major importance and possible benefit e the proposed changes under present consideration the Code of Federal Regulations, Title 20 Chapter 3 Part 405 (Medicare Regulations Pertaining to Independent Laboratories) The two proposed changes that may be of interest are as follows

Section 405 1315 ( ) Standard. Technologist—Duties. (An additional paragraph describing cytotechnologist' authority has been proposed)

(4) an individual who qualified as a cytotechnologist under Section 405 1315(b) (6) following, may supervise technicians and trainees only in the specialty of Cytology

Section 405 1315(b) Standard Technologist—Qualifications (The deletion of the existing paragraph (6) has been proposed with the replacement of a paragraph describing the qualifications of cytotechnologists)

(6) each cytotechnologist shall possess a current license as a cytotechnologist issued by the State if such licensing exists, and (i) shall have successfully completed two years in an accredited college or university with at least twelve semester hours in biology courses pertinent to the medical sciences and must have received 12 months of training in school of cytotechnology approved by the Council on Medical Education of the American Medical Association or (ii) received six months formal training in school of cytotechnology approved by the Council on Medical Education of the American Medical Association and six months of full time experience in cytotechnology in a laboratory acceptable to the Pathologist who directed such formal six months training, or (iii) prior to January 1 1969 shall have been graduated from High School, completed six months of training in cytotechnology in laboratory directed by pathologist or other physician recognized as specialist in cytology and completed two years of fulltime experience in cytotechnology."

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ERRATA

The Editors regret that the incorrect photographs were published in the article by Dr P Leppiluoto entitled "The Etiology of the Cocci Type 'Streptokokkentyp' Vaginal Smear Vol 15 pp. 211–212, 1971 (May–June issue)

The correct figures appear below

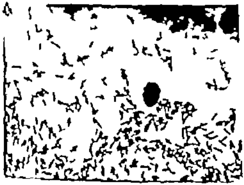

Fig 1 To replace Fig 1 on page 211 Acta Cytologica, May–June 1971

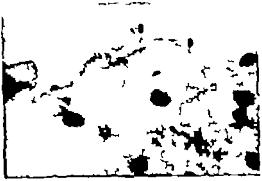

Fig 2 To replace Fig 2 on page 211 Acta Cytologica, May June 1971

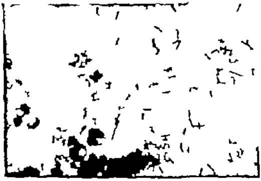

Fig 3. To replace Fig 3 on page 212, Acta Cytologica, May June 1971

In the article entitled The Quinacrine Fluorescence Method of Y-Chromosome Identification, pp 452–454 September October issue of this Journal, that portion of the legend for Figure 1 which reads "More bright spots in the nucleus near eight clock should read "Note bright spot in the nucleus near eight clock.

6) For additional details write to INTERNATIONAL ACADEMY OF CYTOLOGY Committee on Registration and Licensure, 5841 South Maryland Avenue (HM 449) Chicago Illinois 60637 U S A.

Discussion

Kaufmann pointed out that while the present method of testing was not satisfactory, a so-called "peer review" had its pitfalls as well. The principal pitfall is a possible bias that may be legally considered as collusion. Dr Kaufmann further pointed out that no method of external quality control could be considered a substitute for internal quality control. If it were possible to establish such a system based on cytology only without reference to biopsy material, this would be ideal.

Kass raised the question whether it would be possible to substitute the method presently used n New York and Wisconsin (sets of slides sent o the laboratory to be tested) by another method.

Power pointed out that this subject was under scussion by the Subcommittee on cytology and tology of the College of American Pathologists oidance of bias is essential and therefore a del must be designed that will test not only laboratory performance but also the evaluation usefulness of the testing program itself.

Ward suggested that there was a significant difference between licensure by a governmental agency that may be controlled by a legal action and a voluntary acceptance by a laboratory of a recognition by an international body such as the International Academy of Cytology

Dr Thomas R *Simon* (Syracuse, New York) reported that he had the experience of seeing 3 of his very good screeners fail the slide examination This is largely due to poor definition of what is and what is not cytologically suspicious

A *participant* suggested that double screening should prove the best control, just as every patient should be examined by 2 doctors.

Kaufmann agreed with this thought. While there are readily recognized criteria in biochemistry and microbiology there are no such criteria in cytology In essence we are learning on an experimental model.

Dr John K. *Frost* (Baltimore) pointed out that rescreening should not be confused with review or rechecking In his experience physicians are more apt to use rechecking or pattern recognition and not true rescreening

Dr *Ortega* (San Francisco) as a pathologist in private practice was concerned with cost of medical care If double screening were required the cost of cytology would increase immensely He expressed the hope that the American Society of Cytology will become instrumental in providing guidance to pathologists and laboratories as to how to conduct and implement internal control.

Moderator Koss: The panel agreed on the following tentative conclusions and recommendations.

1 Laboratory approval should be supplemented by internal and external quality control on routine bases.

2 Assistance should be offered to all approved laboratories to set up an internal quality control system. This assistance may take the form of actual material or of know how guidance

3 Pathologists and cytotechnologists alike should be encouraged to take postgraduate courses and workshops whenever possible.

4 A uniform system of reporting would assist in compiling plans for the future

The moderator thanked the panelists for reviewing a most difficult topic in a most interesting fashion

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ANNOUNCEMENTS

Norsk Forening for Klinisk Cytologi (Norwegian Society of Clinical Cytology)

The elected officers of the society are

President:	Karl Høeg, M D F.I.A.C.
Vice President:	Arne Serck Hanssen, M D M.I A C.
Secretary Treasurer	Peter B. Lexow M.D
Council Members	Else Mamner C.T (I.A.C.) Ruth Langholm, M.D.

The Society has four meetings during the year The last annual meeting on June 3rd 1971 was joint meeting with group of American doctors and cytotechnologists attending post-congress tour after the International Cytology Congress in London, May 1971

The medical program at this meeting was the following

P Kolstad	Gynecologic cancer in Norway Organization and treatment.
H Bratten, K. *Høeg and K. Nordbye*	*Multiple screening for gynecologic cancer*
A Serck-Hanssen	Urinary cytology in tumors of renal pelvis.
K Nordbye	Tissue culture studies from the human uterine cervix in pre-invasive and invasive carcinomas.
S Rehem d y	Cell kinetics in malignant tumors.
E. Lange and K. Høeg	Cytologic typing of lung cancer A correlation between cytology and histology

Three Norwegian cytologists have in May 1971 successfully passed the I.A.C. Registry Examination for cytotechnologists

Mrs Else Mamner C.T (I A.C)
Mrs. Else Lunde, C.T (I A C.)
Mrs Sidsel Berit Nielsen, C.T (I A.C)

Laboratory Certification by the International Academy of Cytology was in May 1971 issued to

Cytology Laboratory directed by Karl Høeg, M D F.I.A C.,
Department of Pathology
The Norwegian Radium Hospital,
Oslo, Norway

Indian Academy of Cytologists

The First Meeting of the Indian Academy of Cytologists was held on September 20-21 1971 in New Delhi, India.

Prof. P N Wahi, F I.A C President
Prof. Usha K Luthra, F I.A C Secretary General

The program consisted of Nomenclature and Diagnostic Criteria of Cervical Lesions Preceding Invasive Carcinoma with Dr Usha K. Luthra as moderator a Seminar on Hormonal Cytology with the moderator Dr S. M Shahani. The third session consisted *of Organization of Cytological Laboratory with Dr* Meera Alkat acting as moderator and the fourth session consisted of a Diagnostic Cytology Seminar and proferred papers.

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BOOK REVIEWS

Chemistry and Biochemistry Of Amino Acids, Peptides and Proteins Volume 1. Edited by Boris Weinstein. Publisher Marcel Dekker Inc. New York, 1971

The attention of those among the readers of *Acta Cytologica* interested in protein chemistry and the chemistry of amino acids and peptides is directed to the interesting but loose collection of surveys on recent developments brought together and edited by Boris Weinstein. The book is definitively for the specialist.

G. F. Bahr M.D., F.I.A.C.

The Permeability of Natural Membranes. Edited by Hugh Davson and J. F. Danielli. Published by Hafner Publishing Company, Inc. Darien, Connecticut 1970

The publishers of *The Permeability of Natural Membranes* are to be thanked for bringing a classic back to the book market, which long had become *unavailable!* Davson and Danielli's book impresses again by its wealth of ideas and data, presented in the original edition of 1952.

Take for example the "active patch" theory of membrane specificity which, based on studies with erythrocytes, dealt with limited areas of cell membrane *specifically permeable to certain compounds.* Recent advances in electron microscopic preparation techniques and painstaking observations seem to render visible support to this early concept. The preferred interpretation of the general structure of cellular membranes, namely that of two hydrophobic lipid layers applied to opposite sides of a protein layer has its roots in this volume.

G. F. Bahr M.D. F.I.A.C.

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Norsk Forening for Klinisk Cytologi (Norwegian Society of Clinical Cytology)

The elected officers of the society are

President	Karl Høeg, M D F.I.A.C.
Vice President:	Arne Serck Hanssen, M D M.I.A C
Secretary-Treasurer:	Peter B. Lenow M.D
Council Members	Else Manner C.T (I A.C.) Ruth Langholm, M.D

The Society has four meetings during the year The last annual meeting on June 3rd 1971 was join meeting with group of American doctors and cytotechnologists attending post-congress tour after the International Cytology Congress in London, May 1971

The medical program at this meeting was the following

P Kolstad	Gynecologic cancer in Norway Organization and treatment
H. Iversen, K. Høeg and K. Nordbye	Multiple screening for gynecologic cancer
A. Serck-Hanssen	Urinary cytology in tumors of renal pelvis.
K. Nordbye:	Tissue culture studies from the human uterine cervix in pre invasive and invasive carcinomas
S Refsum d y	Cell kinetics in malignant tumors.
E. Lange and K. Høeg	Cytologic typing of lung cancer A correlation between cytology and histology

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Sociedade Latino-Americana de Citologia

The Sociedade Latino-Americana de Citologia will hold the 4th Congress LATINO AMERICANO DE CITOLOGIA in Rio de Janeiro, G B Brazil July 1973 For further details contact Dra Dulce Castellar Sociedade Latino-Americana de Citologia 16 Lauro Mueller 701 Rio de Janeiro Brasil

Canadian Society of Cytology

At the last annual business meeting held in Saskatoon Saskatchewan in June 1971 the following officers were elected

Chairman Dr Ann Worth, British Columbia Cancer Institute Vancouver B C.

Past Chairman Dr Yvan Boivin Hotel Dieu de Montreal, Montreal, Que

Vice Chairman Dr H D Steele Kingston General Hospital, Kingston Ont.

Secretary Treasurer Dr R H Curry Ottawa Civic Hospital, Ottawa Ont

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National Editor for ACTA CYTOLOGICA (Canada) Dr A Meisels, Laval University Quebec City Que

Sociedad Argentina de Citologia

The Executive Committee of the Sociedad Argentina de Citología for the period 1971–1972 has been designated as follows

Presidente	Marcos Peluffo
Vicepresidente	Gustavo A. Schuckendantz
Secretario General	Francisco Laphitz Flores
Secretario de Actas	Juan P Emina
Tesorero	Oscar J Mezzano
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4 Vocal Titular	Jacobo Rotman
1 Vocal Suplente	Carlos Arrue Gowland

2° Vocal Suplente	María Mercedes Gamboni
3° Vocal Suplente	Alfredo Wainer
4 Vocal Suplente	Ofelia T de Estevez

Second Tutorial on Human Chromosomes and Chromatin

January 12–15 1972

The International Academy of Cytology and the American Academy of Reproductive Medicine in cooperation with the Center for Continuing Education of the University of Chicago will sponsor a four-day course on “Human Chromosomes and Chromatin.”

Classical and advanced techniques for the identification of chromosomes and the study of chromatin will be demonstrated. Theory results, and where appropriate clinical significance will be presented.

The following topics will be presented and or demonstrated Karyotyping clinical cytogenetics including amniocentesis, linkage and meiotic studies, quinacrine fluorescence technique satellite DNA detection X, Y-chromosome identification, cell hybridization, critical point drying of chromosomes and chromatin, quantitative light and electron microscopic measurements, karyotyping by computer

The Tutorial intends to review current knowledge and to demonstrate the more readily available techniques to cytologists, pathologists, pediatricians, anatomists and others interested in the relation of the properties of chromosomes and chromatin to disease.

The Registration Fee is $250.000 (payable to the University of Chicago) Housing and meals are not included in this fee.

An application form may be requested by writing to Second Tutorial on Human Chromosomes and Chromatin University of Chicago Center for Continuing Education, Chicago, Illinois 60637

Fifth Tutorial on Clinical Cytology

January 30–February 6 1972

The International Academy of Cytology and the American Academy of Reproductive Medicine in cooperation with the Center for Continuing Education of the University of Chicago will sponsor a 7-day course on Clinical Diagnostic Cytology. The course deals with *gynecologic cytology (5 days) and aspiration biopsy cytology* (2 days) and is for pathologists, residents preparing for the American Board examinations (American Board of Pathology and American Board of Obstetrics and Gynecology) clinicians interested in diagnostic cytology and the experienced cytotechnologist.

The Tutorial will be held at the Center for Continuing Education of the University of Chicago 1307 East 60th Street, Chicago, Illinois. The Center is a fully equipped residential center where Registrants may live during the course, if hotel space permits.

Housing

Tutorial Registrants requesting accommodations at the time of their registration will be housed either in the CENTER FOR CONTINUING EDUCATION of the *University of Chicago, 1307 East 60th Street, Chicago 60637* (Telephone 312 288-2500) or in hotels or motels in the immediate vicinity of the Center If your room is in another motel or hotel in the area, transportation will be provided to the Center for all sessions.

Registrants should receive confirmed room reservations six (6) weeks prior to the onset of the Tutorial. If not, please write or call Mrs. Eleanor OLIVER, Center for Continuing Education University of Chicago 1307 East 60th Street, Chicago, Illinois 60637 (Telephone 312 288-2500) As a general rule, accommodations are *confirmed at the time of the acceptance of the registration.*

Fees

The fee for the full course is $300.00. Housing and meals are not included in this fee. Checks should be made payable to the "University of Chicago.

Application Form

An application form and further details may be requested by writing to

Fifth Tutorial on Clinical Cytology
University of Chicago
5841 South Maryland Avenue—HM 449
Chicago, Illinois 60637
(Telephone 312 947 5343)

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Subject Index

American Society of Cytology 18th Annual Meeting
  presidential address, 327
  proceedings, 329–413

Barr bodies (letters to the editor) 325
Bilharziasis, gynecologic, in cytologic smears, 482
Biopsy
  aspiration, neck tumors, 25
  colposcopically directed false negative cervical cytology 292
  correlation with direct vision brushing cytology 375
  transthoracic needle diagnosis of lung tumors (letter to the editors) 209
Bladder irrigation specimen, exfoliative cytology 385
Book Reviews
  Automated Cell Identification and Cell Sorting, 520
  Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins, 578
  Permeability of Natural Membranes, 578

Calcified bodies, intrauterine device, 473
Cancer peripheral, lung, early diagnosis, 460
Candida sp. squamous cell changes, 379
Carcinoma
  breast
    cytologic differentiation, 400
    sex chromatin as prognostic factor 372
  cervical in situ, incidence in Iraq, 171
  in situ
    cell pathology 513
    tissue pathology 513
  palatal, DNA values from cytology 559
  renal cell, cytologic diagnosis, 31
Carotid body tumors, cytologic findings, 25
Cells
  abnormal, test strategy for detection, 553
  analysis objectives, 1
  atypical,
    in pleural fluid of systemic lupus erythematosus patients, 337
    in spermatocele fluid (letter to the editors) 210
  buccal, X-chromatin frequency 294
  cancer
    effect of 3-[illegible] (letter to the editors) 125
    TV brushing technique for diagnosis, 460
  cervical ultrasonic dispersal, 163
  classification, automatic, 158
  comet, in urinary sediment smears, 303
  computer recognition, 147
  decoy, in urinary sediment smears, 303
  detection automatic 158
  exfoliated, morphology 128
  malignant, in cerebrospinal fluid, 565
  mononuclear transformation in cerebrospinal fluid, 545
  plasma, hydroquinone tetrazolium reductase reaction, 63
  population patterns in automated cytology 444
  prescreened
    marking in automated cytology 422
    sorting in automated cytology 422
  properties, quantitative measures, 13
  recognition
    by gray value analysis, 284
    in digitized images, 284
  renal carcinoma cytologic diagnosis, 31
  senescence (letter to the editors) 325
  squamous, changes associated with *Candida* sp. 379
  vaginal, X-chromatin frequency 294
Cervix, uterine, sex chromatin in cells, 297
Chromatin, sex
  in vitro observations, 297
  prognostic factor in breast carcinoma, 372
Chromosomes, findings in leukemic reticuloendotheliosis, 173
Colpocytology hormonal, comparison with endometrial histology 329
Condenser crushing
  for cytologic studies, 419
  for photometric studies, 419
Crazing of mounting medium, antioxidant inhibition (letter to the editors), 419
Cytochemical determinations
  dry mass, 499
  protein content 499
Cytodiagnosis
  lymphadenopathies, imprint method, 123
  tumors of head and neck imprint method, 123
Cytogram, cervico-vaginal, folate deficiency 50
Cytologic changes, sequential, in herpesvirus, 455
    cell population patterns 444
Cytology
  automated
    cell analysis objectives, 1
    descriptors, 87
      definition, 116
    marking prescreened cells, 422
    parameters, 87
    preparation requirements, 216
    prescreening, 236
    quantitative measures of cell properties, 13
    sample collection, 216
    sorting prescreened cells, 422
  cervical, incidence of false negative, 292
  differentiation of breast carcinoma, 400
  direct vision brushing
    correlation with biopsy 375
    of malignant tumors, 375
  exfoliative
    proficiency testing program
      development, 34
      evaluation, 468
    urinary bladder
    Wilms' tumors, 539
  first symposium, abstracts, 182
  genital, in feminizing tumors of ovary 334
  ileal conduit urine 506

  laboratories
    certification, 56.
    licensure, 562
    quality control 56.
  peritoneal fluid and ovarian teratoma, 306
  proficiency testing program, 351
  quality control method, 54
  vaginal, and mycoplasma 310
  vulvar 57
  W.H O nomenclature, 318
Cytometry cytochemical determinations, 499
Cytophotometry DNA, tumor diagnosis, 154
Cytotechnology
  careers 317
  Hartford Hospital School 347

Deoxyribonucleic acid values from cytology 559
Diagnosis
  cytologic
    peripheral lung cancer 460
    tissue repair 133
  lung tumors, transthoracic needle biopsy (letter to the editors) 209
Dipetolenema perstans in cervical smears, 479
Double-minutes aberration in rhabdomyosarcoma 4
Dysplasias, uterine cervix
  cell pathology 513
  tissue pathology 513

Erythematosus, systemic lupus atypical cells in plural fluid 357
Estrogens synthetic, comparative potential 167
Ethinylestradiol comparison with mestranol, 167
European Federation of Cytology Societies
  first symposium on cytology abstracts 182

Fluid, cerebrospinal
  malignant cells in, 363
  mononuclear cells 345
Fluorescence
  acridine orange machine characterization of leukocytes, 289
  quinacrine in Y-chromosome identification, 452
Folate deficiency in cervico-vaginal cytograms 50

Genital tract female cytologic diagnosis of tissue repair 133

Hartford Hospital School of Cytotechnology 347
Herpesvirus, sequential cytologic changes, 455
Histology endometrial, comparison with hormonal colpocytology 329
Hydroquinone tetrazolium, reductase reaction, 85

Imprint method, cytodiagnosis
  lymphadenopathies, 123
  tumors of head and neck 123
Incrustation, intrauterine device 473
International Academy of Cytology fellows and members, 64
Intrauterine device, calcified bodies, 473

Iraq, incidence of cervical carcinoma in situ, 171
Irrigation, urinary bladder specimens, exfoliative cytology 383
3 Isothiazolone effect on human cancer cells (letter to the editors) 325

Larva microfilial, stages in gynecologic smears, 476
Leukemia, lymphocytic, computer recognition of cells, 147
Leukocytes, machine characterization by acridine orange fluorescence 289
Lymphadenopathies, cytodiagnosis, imprint method, 123

Macrophages pulmonary eosinophilic extracytoplasmic processes, 363
Megakaryocytes, circulating by age and sex, 410
Melanoma vaginal, primary malignant, 179
Mestranol, comparison with ethinylestradiol, 167
Morphology exfoliated cells, 128
Mycoplasma, and vaginal cytology 310

Nucleus, aberration in malignancy associated changes 553

Presidential address 18th Annual Meeting American Society of Cytology 327
Processes eosinophilic extracytoplasmic in pulmonary macrophages, 363
Proficiency testing program
  computerized, New York State
    development, 34
    evaluation 468
  Wisconsin, 351

Reductase reaction hydroquinone tetrazolium, 85
Reticuloendotheliosis, leukemic chromosomal findings 173
Rhabdomyosarcoma chromosomal aberration, 42

Sex, prenatal determination, 316
Smears
  buccal, malignancy associated changes, 46
  cervical
    automatic classification of cells 138
    automatic detection of cells, 138
    Dipetolenema perstans 479
  cytologic
    gynecologic bilharziasis, 482
    preparation from solid tumors, 143
  gynecologic microfilarial larval stages, 476
  Papanicolaou, squamous cell changes, 379
  urinary sediment, comet or decoy cells, 301
  vaginal
    cocci type etiology (letter to the editors) 211
    Streptokokkentyp etiology (letter to the editors) 211
Spermatocele atypical cells in fluid (letter to the editors) 210
Stain, fading, antioxidant inhibition (letter to the editors) 419

Teratoma ovarian, peritoneal fluid cytology 306
Test statistic, detection of abnormal cells, 533

- Tissue repair cytologic diagnosis, 133
- Tumors
  - carotid body cytologic findings, 25
  - diagnosis
    - ascitic effusions, 154
    - pleural effusions, 154
  - feminizing, of ovary 334
  - head, cytodiagnosis, imprint method, 123
  - lung, transthoracic needle biopsy (letter to the editors), 209
  - malignant, direct vision brushing cytology 375
  - neck
    - aspiration biopsy 25
    - cytodiagnosis, imprint method, 123
  - solid, preparation of cytologic smears, 343
  - Wilms exfoliative cytology 339
- TV-brushing technique fresh cancer cells, 460
- Ulcer benign gastric, morphology of exfoliated cells, 128
- Urine Ileal conduit, cytology 506
- W.H.O. nomenclature in cytology 318
- X-chromatin, frequency in buccal and vaginal smears, 294
- Y-chromosome, identification, quinacrine fluorescence method, 452

Acta Cytologica
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Vol 15, No 6
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Contributors to Volume 15

Adams, Lawrence R., 289
Albrecht, Robert, 468
Alonso Patricia, 209
Amatulli, Joseph M 365
Arendzen, J H 316
Atkin N B., 419
Aust, Rudolf, 123

Bahr Gunter F 1 87 147 284 325 499 533
Barord, Mario 173
Bartels, Peter H 147 284 533
Bellamy John C., 147 284
Berry Ann, 482
Bhattacharya, Prodyot, 533
Bibbo Marluce, 133
Borgaonkar Digamber S 45
Browell, B. M., 385
Bunnag Bill, 306

Campbell James A 297
Cano-Valle Fernando 209
Chu, Elizabeth W 306
Clarke Edna, 351
Cleary Brendan K., 163
Cohen, Martin, 54
Collins Doris N 34 468
Crabbe J G S. 303
Crow Wilfred D 325

Dago Carla 410
Davis, Robert H. 306
de Borges, Rosa, 476
DelVecchio Pasco R., 506

Engzell, U 45
Evans, David M D 163

Fennell, Robert H., 377
Finch, Robert R. 46 553
Forni, Alessandra, 173
Franzén, S., 45
Freni, S C., 154
Frost, John K., 419
Fullmer Cyril D 363

Glenn Fred L., 365
Goldston, William R., 334
Gondos, Bernard, 292
Granberg Ingrid, 4
Gray R. L., 385
Green, Sidney 325
Griswold, Anne L., 347

Hajdu, Eva O 31
Hajdu, Steven I 31 339
Harris, M. J 385
Hattori, Shoji, 460
Heller Carol 379

Hernández, F., 85
Highman, Wilma J 473
Hollander David H 419 452
Hoovis, E., 57
Horai, Takeshi 460
Hoyt, Virginia, 379
Huisjes, H J 316
Hutson, Yolande, 357

Iglesias, J R., 85
Inhorn, Stanley L., 351

James J 154
Jarkowski Thaddeus L., 147 284
Jenny J 182
Johnston, William W 334 365

Kalin, Eloise W 294
Kamentsky Louis A., 289
Karpas, Charles M., 410
Kaufmann, William, 34 468
Keebler Catherine M., 133
Kelley Susan, 357
Ketcham Alfred S 306
Kirsner Joseph B., 128 375
Klaus, Hanna, 50
Klionsky Bernard, 54
Koss, Leopold G 31 379
Kubba, Karama, 171

Layton Jack M 147 284
Leonard, Nelson, J 325
Leppäluoto, Pentti A., 11
Linthicum, C. Milton, 179

Malmgren, Richard A., 506
Mårdh, P A., 310
Mark, Joachim, 4
Matsuda, Minoru, 460
McGarry Paul 357
McGowan, Larry 306
McKinstry Donald W 325
Montgomery Martha S 334
Morrow J W 385
Murad, Tariq M 400

N uss, Jean M., 347
Niebergs, Herbert E 513
Nishihara, Hiromu, 460
Norquist, Leslie Anne 343

Oppenheim, Abraham, 410
Ostergard, Donald R 292
Otis, Richard D 347

Pacifico Emma, 173
P pe D H 559
Parry Janet S 163

Pfitzer P 339
Pozza, Louis, 410
Plaz, Luis I 294
Ponce de Leon, Hector 209
Prolla, Joao C., 128, 375
Pop, F J A 154

Reyniak, J Victor 329
Rundosh, Poonappen, 329
Randall, T 455

Sinds, Rune, 543
Savino, Angela, 31 372
Schwed, Abraham L., 343
Scherma, C. P 383
Sedlis, Alexander, 329
Sharma, Santosh D 479
Sobrero, A. J 57
Soloway Mark S., 506
Stahle, Jan, 123
Stenkvist, Björn, 123
Stone, Dorothy 329
Stormby N 310
Stupnicki, Romuald, 167

Teplitz, R. L., 455
Teter Jerzy 167
Tozzini, R., 57
Trussell, Richard R., 479
Tucker J H 138
Tytun, Alex, 410

Unitt, Mary 210

Valco, Z., 455
von Haam, Emmerich, 46

Wager Sharon, 210
Walker Kenneth R., 363
Wentworth, Paul, 210
Wert, William L., 325
Weström, L., 310
Wied, George L., 1 87 133 147 284 499 533
Wilbanks, George D 297
Williams, A. Roy 163

Xavier Rogerio G 128 375

Yoshii, Yuri, 375

Zajicek, J 25
Ziegler Otto, 479
Zadowsky J 182